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FROM THE DEPARTMENT OF MEDICAL CHEMISTRY, UNIVERSITY OF HELSINKI

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PREFACE

This investigation was carried out at the Department of Medical Chemistry, University of Helsinki. I am very much indebted to the Head of this Department, my greatly honoured teacher Professor P. E. Simola, M. D., Ph. D., for his kind interest and expert advice in connection with the investigation, and for his valuable suggestions in the preparation of the manuscript. I wish also to thank him for his kindness in placing the facilities of the Department at my disposal.

Further I thank my colleagues in the Department for their helpful attitude. My thanks are due especially to Dr. Martti Koivusalo, M. D., for the many stimulating discussions which, because of his wide knowledge of the subject, were of great importance for the progress of this study, and to Dr. Tatu Miettinen for willing assistance given to me.

I express my sincere gratitude to all persons who in any way have shown an interest in this investigation and have promoted it with welcome advice.

I am also indebted to Juhani Virkkunen, M. Sc., for the statistical treatment of the data.

In this connection I wish also to thank Miss Aune Priha, Mrs. Hilkka Rönkkö and Mrs. Margit Venesjärvi for their skillful technical assistance in various phases of the work.

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Helsinki, June 1958.

Tapani Luukkainen

CONTENTS

INTRODUCTION

The rapid advances in endocrinology have increased our knowledge of the effects of hormones on the animal organism. However, the mechanism of hormonal action and especially the mode of hormonal regulation of cell metabolism are still obscure. We are nevertheless able to anticipate that these are very complex; several enzymes in different cell components change in an individual manner under the influence of hormones.

The acetylation of aromatic amines has been the subject of a large number of experiments, and information accumulating in this area has led to the nearly complete clarification of the mechanism of this reaction. Since the acetylation of aromatic amines might reflect other acetylation reactions and since some of the acetate reactions have been shown to be under hormonal control, it may be of interest to study the hormonal regulation of this enzymic phenomenon. It is further known that arylamine acetylation in the animal organism can be followed as well under *in vivo* as under *in vitro* conditions.

In the animal body, administered aromatic amines, such as *p*-aminobenzoic acid, are also conjugated with radicals other than acetyl. The short survey in the following chapter is concentrated only on points relating to acetylation. Other forms of conjugation will be briefly touched upon later and the reader is therefore referred in this respect to more detailed surveys on the subject (*e.g.*, Stekol 1941, Handler and Perlzweig 1945, Wright and Tavormina 1954).

In this laboratory, studies on the oxidation of pyruvate and on the metabolism of acetate have occupied a central position among the problems investigated during nearly three decades

(*e.g.* SIMOLA 1932, 1938 a, 1938 b, 1939, 1944, SIMOLA and KRUSIUS 1939, HALLMAN 1940, KRUSIUS 1940, SIMOLA and ALAPEUSO 1943, KINNUNEN 1946). The present investigation is a part of this research work. The object of the experiments described in the present report was to study both *in vivo* and *in vitro* the regulation of the acetylation of *p*-aminobenzoic acid by the sex glands and the various factors associated with this reaction in the rat organism.

REVIEW OF THE LITERATURE

In 1893 Cohn isolated a mixture of acetyl-*p*-aminobenzoic acid and *p*-nitrobenzoic acid from the urine of rabbits which had been given *p*-nitrobenzaldehyde. He thus demonstrated that the organism is capable of producing acetyl groups and of converting aromatic amines to their acetylated derivatives. It was demonstrated by Neubauer and Warburg (1910) that the formation of acetyl groups and acetylation occur in the liver, since acetylphenylaminoacetic acid was formed on perfusion of the liver with phenylaminoacetic acid. In the same year Knoop (1910) observed the excretion of acetylated benzylalanine in the urine after the ingestion of benzylpyruvic acid. His investigation was evidence in support of the opinion concerning the acetylation capacity of the organism and of its ability to form amino acids from keto acids and ammonia. Four years later Ellinger and Hensel (1914) gave *p*-aminobenzoic acid to rabbits and found that a part of it was regularly excreted in the urine as acetylated *p*-aminobenzoic acid. Later it was demonstrated that acetylation of aromatic amines also occurs in man (Cerecedo and Sherwin 1923) and in most vertebrates (see, *e.g.*, Krebs, Slykes and Bartley 1947, Williams 1949).

In the following survey the investigations which have dealt in greater detail with the acetylation of aromatic amines are grouped according to the subject of investigation and each group is discussed separately.

ACETYL PRECURSORS IN THE ACETYLATION OF AROMATIC AMINES

Before the use of isotopes in research work it was possible to draw only indirect conclusions concerning the participation of various substances as source of acetyl groups in the acetylation of aromatic amines in the organism.

In experiments carried out *in vivo*, aromatic amine was fed to the control animals, and the aromatic amine and the substance being tested were given to the experimental animals. If acetyl groups were formed in the organism from this substance, a possibility exists that some of these groups would be conjugated with the aromatic amine and a larger amount of the

latter would therefore be acetylated in the experimental animals than in the controls.

Hensel (1915) was the first to conduct these experiments, and he found that acetate and pyruvate increase acetylation. His results were confirmed by Harrow and co-workers (Harrow, Power and Sherwin 1927, Harrow, Mazur, Borek and Sherwin 1934), who observed that also after the administration of tyrosine, ethylacetate, β-hydroxybutyric acid, glutamic acid, hexoses, glycerol and lactate the excretion of acetylated *p*-aminobenzoic acid conjugates in the urine is greater in the experimental animals than in the controls. The acetylation of sulphonamides also was increased after acetate (James 1939). According to other investigators the administration of acetate may have an inhibitory effect on the acetylation of aromatic amines (Doisy and Westerfeld 1943, Martin and Rennebaum 1943). It was shown by Charalampous and Hegsted (1949) that the administration of acetate, pyruvate and lactate did not affect the degree of acetylation of *p*-aminobenzoic acid in either direction. Glycine and malic acid, when given simultaneously with a large oral dose of *p*-aminobenzoic acid (200 mg per kg), increased the amount of acetylated conjugates in the urine of rabbits (Venkataraman, Venkataraman and Lewis 1950).

In vitro experiments, on the other hand, have given more uniform results. The use of larger amounts of aromatic amines and precursors in relation to the amount of tissue is possible in these experiments and the technique is more reliable, since it is possible to use liver tissue from the same animal for both the experimental and the control groups and thus to eliminate errors due to individual variations. Klein and Harris (1938), Lipmann (1945), Kinnunen (1946) and Phillips and Anker (1957) made a similar observation that acetate caused the most powerful increase in the formation of acetylated aromatic amines. However, only by the use of isotopically labelled substances and the demonstration of radioactive acetate in the acetylated aromatic amines is it possible to determine definitely that acetyl groups have actually been formed from the substance under examination for acetylation. In this manner it was possible to demonstrate beyond doubt the participation of various substances as acetyl donors to aromatic amines (*e.g.*, Bernhard 1940 a, b, Bloch and Rittenberg 1944, 1947, Anker 1950).

EFFECT OF VITAMINS IN THE ACETYLATION OF AROMATIC AMINES

Martin and Rennebaum (1943) were the first workers to study the acetylation of sulphanilamide in deficiency conditions of thiamine and riboflavin. They observed that the 24-hour excretion of acetylated sulphanilamide in the urine of animals deficient in these vitamins was lower

than that of control animals. The acetylation capacity of liver homogenates of pigeons kept on a thiamine deficient diet has been found to be increased by the addition of cocarboxylase (LIPMANN 1945). In our laboratory KINNUNEN (1946) was able to raise *in vivo* and *in vitro* the depressed acetylation power observed in rabbits during the winter months by thiamine injections to the animals. Thiamine added *in vitro* and riboflavin injected *in vivo*, on the other hand, had no effect on the depressed acetylation power of the rabbit liver. In man the administration of thiamine had an increasing action on the acetylation of sulphonamides (FORNI 1948). In thiamine and riboflavin deficiency states the capacity of rats to acetylate *p*-aminobenzoic acid was decreased when a dose of 1—2 mg was used, but no difference was demonstrable when the dose was 10 mg or more; the administration of acetate did not raise this decreased capacity for acetylation (RIGGS and HEGSTED 1949). By contrast to these results, SHILS, SELIGMAN and COLDWATER (1949) and SHILS, ABRAMOWITZ and SASS (1950) were unable to demonstrate any significant difference in the capacity of thiamine, riboflavin or pyridoxine deficient rats to acetylate sulphanilamide as compared to control rats.

In experimental animals deficient in pantothenic acid, which is a part of coenzyme A, the capacity to acetylate aromatic amines is decreased (RIGGS and HEGSTED 1948, 1949, 1951, SHILS *et al.* 1949, 1950, RIGGS and CHRISTENSEN 1951, DUMM and RALLI 1951). Human subjects deficient in pantothenic acid (in the »burning feet syndrome») acetylate a smaller amount of *p*-aminobenzoic acid than healthy persons (SARMA, MENON and VENKATACHALAM 1949). Also this decrease in acetylating power due to pantothenic acid deficiency was significant in comparison to control animals only when à given dose of *p*-aminobenzoic acid was used (RIGGS and HEGSTED 1949) and it disappeared when benzoate was administered simultaneously with the *p*-aminobenzoic acid (RIGGS and CHRISTENSEN 1951). In addition, a decrease in the capacity to acetylate *p*-aminobenzoic acid has been observed in scorbutic animals (BELAVADY and BANERJEE 1954).

EFFECT OF HORMONES

Insulin was the first hormone observed to have an effect on the acetylation of aromatic amines. HARROW, MAZUR and SHERWIN (1933) and HARROW *et al.* (1934) demonstrated that insulin injections resulted in a stimulation of the acetylation of *p*-aminobenzoic acid in rabbits. On the other hand, HEIN-SEKULA and ROLLER (1945) found the acetylation of sulphonamides decreased in human diabetes and ascribed it to a deficiency of acetyl groups in this disease. Similarly in alloxan-diabetic rats (CHARALAMPOUS and HEGSTED 1949) the percentage of acetylated *p*-aminobenzoic acid conjugates in the urine was lower than in non-diabetic control animals

when the *p*-aminobenzoic acid was given in a dose of 0.5—3.0 mg (3.5—22 μmoles). This difference in the acetylation of *p*-aminobenzoic acid disappeared when the diabetic rats received insulin or large doses — in relation to the aromatic amine — of either citrate, α-ketoglutarate, succinate, fumarate, malate, oxaloacetate, diacetyl, acetylphosphate or adenosine triphosphate. Similarly, in liver slices from diabetic rats less acetylated sulphanilamide was formed *in vitro* than in liver slices from non-diabetic rats (FRIESEN 1956). GERSHBERG and KUHL (1950), on the other hand, were unable to demonstrate any difference in the acetylation of *p*-aminobenzoic acid in diabetic and non-diabetic humans. NIKKILÄ and KERPPOLA (1957) observed that the excretion of acetylated *p*-aminobenzoic acid conjugates in the urine was higher in patients with untreated diabetes during acidosis than in normal persons or treated diabetics. In experimental ketosis in rabbits, HOLLMANN and DOMAGK (1953) observed no change in the acetylation of aromatic amines.

In studying the effect of the thyroid gland on sulphanilamide acetylation, FRAENKEL-CONRAT and GREENBERG (1946) observed that 30 per cent of the sulphanilamide excreted in the urine by female rats after addition of dried thyroid gland to the diet was acetylated sulphanilamide, after thyroidectomy 33 per cent, after thiouracil ingestion 44 per cent, and in controls 39 per cent. In thyrotoxic patients the capacity to acetylate *p*-aminobenzoic acid was also lowered (GERSHBERG, KUHL and RALLI 1950, GERSHBERG and KUHL 1950, VAVREČKA and PETRÁŠEK 1955). However, the addition of thyroxine had no effect on the activity of an enzyme system prepared from pigeon liver (FRANZ and LATA 1953), and tri-iodotyrosine given to rats *in vivo* did not affect acetylation in liver slices *in vitro* (FRIESEN 1956).

A difference between male and female experimental animals in the capacity to acetylate aromatic amines has been demonstrated by some investigators. Of interest is the observation that among rabbits the females have a higher capacity to acetylate sulphonamides than the males both *in vivo* and *in vitro* (KINNUNEN 1946), whereas in rats *in vivo* the males acetylate a larger amount of aromatic amine than the females (DUMM and RALLI 1951, FRANZ and LATA 1953, 1957). This dissimilarity is probably due to differences in the acetate metabolism of herbivores and omnivores. When steroid hormones were added *in vitro* to a sulphanilamide acetylating system prepared from pigeon liver it was observed that acetylation was stimulated by testosterone and to a lesser degree by progesterone, whereas oestrogens had little effect (FRANZ and LATA 1953, 1957). Studies have recently been published by FISHKIN and LATA (1957, 1958) in which they demonstrated that testosterone propionate increases the capacity of female rats to acetylate sulphanilamide, and that oestradiol injected into males decreases the degree of acetylation. The castration of adult male rats (FRANZ and LATA 1957), on the contrary, was found to have no effect.

According to FISHKIN and LATA (1957, 1958) the sex-linked difference was not observed in immature male and female rats. The acetylation power of young male rats was lower than that of adult males. The castration of males immediately after birth decreased to some extent the relative amount of acetylated sulphanilamide when these animals were tested one to two months after the operation. On the other hand, the removal of the sex glands of immature female rats had no effect on tests carried out 31—42 days after the operation.

The rôle of the adrenal glands in the regulation of aromatic amine acetylation is probably rather insignificant. SHILS *et al.* (1949) observed that adrenocortical extracts did not affect sulphanilamide acetylation in rats. According to GERSHBERG and KUHL (1950), the capacity of patients with Addison's disease to acetylate aromatic amines was at the lower limit of normal but was slightly raised by the administration of adrenocortical extracts. Adrenalectomy in rats was found by DUMM and RALLI (1951) not to have any effect in this respect, but in adrenalectomized animals kept on a pantothenic acid deficient diet the acetylation capacity reverted more slowly to normal after pantothenic acid administration than in the non-operated animals. The addition of cortisone to the acetylating system isolated from pigeon liver slightly stimulated the acetylation (FRANZ and LATA 1953). *In vitro* studies of the livers of adrenalectomized rats revealed no difference in acetylation when compared with non-adrenalectomized rats, nor did the acetylation power of rat liver change after the *in vivo* administration of cortisone (FRIESEN 1956).

SITE OF ACETYLATION AND DEACETYLATION OF AROMATIC AMINES IN THE ORGANISM

In their experiments *in vitro*, HARRIS and KLEIN (1938) studied the capacity of rabbit and rat liver, muscle, spleen, kidneys and blood to acetylate sulphanilamide. The intact liver cell was the only preparation capable of acetylating sulphanilamide. The acetylating activity, furthermore, was demonstrable in only a part of the rat livers examined. The liver has been found to be the site of acetylation of aromatic amines also in experiments *in vivo*. STEWART, ROURKE and ALLEN (1939) studied sulphanilamide acetylation in rabbits after the removal of different organs and observed the disappearance of the acetylating power following hepatectomy. Removal of the stomach, intestines, pancreas, spleen and kidneys had no definite effect on the acetylating power. A number of later investigators also regarded the liver as the most probable site of acetylation of aromatic amines (KINNUNEN 1946, KREBS *et al.* 1947, ANKER 1950, HUNG 1953, PHILLIPS and ANKER 1957).

It seems evident, however, that some acetylation also takes place

extrahepatically. For example, the acetylating power of the cat has been observed to disappear only after the simultaneous removal of the liver and the spleen (VAN WINKLE and CUTTING 1940). Acetylation has also been found to occur in some tumour tissues (GOTH 1942), the intestinal wall (BLONDHEIM and KUNKEL 1950) and human and rat erythrocytes (BLONDHEIM 1955). In the kidney no acetylation of sulphanilamide or *p*-aminobenzoic acid was obtained in any of the experiments reported in the literature (HARRIS and KLEIN 1938, KINNUNEN 1946, KREBS *et al.* 1947, PHILLIPS and ANKER 1957, BRAY, FRANKLIN and JAMES 1958).

On the other hand, *p*-aminosalicylic acid was acetylated *in vitro* in kidney slices from the guinea pig, mouse, rat and rabbit in varying amounts compared to hepatic acetylation, according to experiments published recently by LAUENER, HODLER, FAVEZ, DETTWILER, and HADORN (1957). These investigators also observed acetylation of *p*-aminosalicylic acid in the rat spleen, lung and muscle, and by heparinized rat and rabbit blood.

One of the reasons why aromatic amines have been widely used in the study of acetate metabolism is that the reaction has been regarded as irreversible. The investigations of a number of workers have demonstrated that, in fact, acetylated aromatic amines formed in the organism or fed to animals are not decomposed again in the animal body (*e.g.*, VAN WINKLE and CUTTING 1940, STRAUSS, LOWELL, LASHEY and FINLAND 1941, ROBINSON and CROSSLEY 1943, FISHMANN and COHN 1943, KIRCH and BERGEIM 1943, SMITH and WILLIAMS 1948).

On the other hand, KOHL and FLYNN (1940) observed in liver pulp the occurrence of deacylation, which increased with an increasing number of carbon atoms of the acyl group. Results of a similar kind were obtained by KREBS *et al.* (1947), who reported deacetylation *in vivo* and especially *in vitro*. In recent experiments, perfusion of the rat liver with a solution containing iodoacetate resulted in similar although weak deacetylation, which, however, was not demonstrable under physiological conditions (BETTSCHART, KOHN and BOVET 1957).

FORMATION OF ACETYL COENZYME A

Although experiments *in vivo* show best what metabolic events take place in the living organism, it is difficult and frequently impossible to obtain a view of the details of an enzymic reaction. On the other hand, the elucidation of the mechanism of most reactions is possible by *in vitro* methods and especially by using purified enzymes. Accordingly, only the use of *in vitro* experiments will give a detailed picture of the reactions connected with the acetylation of aromatic amines.

HARRIS and KLEIN (1938) were the first to determine the conditions under which the acetyl group conjugated with sulphanilamide *in vitro*. Continuing their experiments, KLEIN and HARRIS (1938) found that the

acetylation of sulphanilamide in slices of rabbit liver was greater under aerobic than under anaerobic conditions. Certain oxidation poisons, such as cyanide and iodoacetate, inhibited acetylation, although the acetyl groups were supplied in the form of acetate. It thus was shown that even with acetate as an acetyl precursor the presence of oxygen was necessary. However, oxygen does not enter into the formal equation of

$$\text{acetate} + \text{sulphanilamide} \rightarrow \text{acetylsulphanilamide}.$$

It was therefore considered likely that during the oxidative processes there was formed some substance that was also indispensable for the acetylation of sulphanilamide.

In the experiments with homogenates of pigeon liver, LIPMANN (1945) demonstrated that acetate was able to act as an acetyl donor also anaerobically if energy was provided in the form of adenosine triphosphate. It seemed probable that under aerobic conditions there was formed adenosine triphosphate, which was required in the »activation of acetate». Of interest is also the observation by KINNUNEN (1946) that under anaerobic conditions pyruvate is a better acetyl donor than acetate, and that the opposite is true under aerobic conditions. It therefore appeared probable that the »activation» of acetyl formed from pyruvate differs from the activation of acetate.

In the investigation cited above, LIPMANN observed sulphanilamide acetylating activity not only in homogenates but also in cell-free extracts of pigeon liver. With aging of the extracts their activity disappeared, which was not an unexpected but rather an expected observation. LIPMANN, continuing his experiments, made the important observation that inactive extracts may be activated by the addition of »Kochsaft», *i.e.*, water in which liver had been boiled. Thus the acetylation of sulphanilamide in liver extracts required — in addition to acetate, adenosine triphosphate and thermolabile enzymes — a water-soluble, thermostable activator: a coenzyme. This new coenzyme, coenzyme A, on which the acetylation was dependent and which proved to be a derivative of pantothenic acid (LIPMANN, KAPLAN, NOVELLI, TUTTLE and GUIRARD 1947) was present in different parts of the animal organism, in micro-organisms, and even in plants (KAPLAN and LIPMANN 1948).

The mechanism of »activation» and the nature of »active acetate» lacked a definite explanation until in 1951 LYNEN, REICHERT and RUEFF observed that the acetyl thio ester of coenzyme A — acetyl CoA — was capable of being an acetyl donor to sulphanilamide without the presence of acetate and adenosine triphosphate.

Subsequent studies demonstrated that acetyl CoA was the »active C_2 fragment» in also other reactions. It also became evident that at least two enzymes were connected with the sulphanilamide acetylation in pigeon liver extracts (CHOU and LIPMANN 1952). One of these enzymes, acetate thiokinase (aceto-CoA-kinase) formed acetyl CoA from acetate, adenosine

triphosphate and CoA, and the other enzyme, arylamine acetylase (aceto-arylamine-kinase) acetylated sulphanilamide in the presence of acetyl CoA.

The reaction activated by acetate thiokinase occurs by steps (Jones, Black, Flynn and Lipmann 1953, Jones, Lipmann, Hilz and Lynen 1953) and adenyl-acetate is probably formed as an intermediate (Berg 1956). Arylamine acetylase, again, is believed to undergo irreversible acetylation by the action of acetyl CoA, and the acetylated enzyme in turn acetylates arylamine (Bessmann and Lipmann 1953). The acetyl CoA formed from acetate is only a small part of the total acetyl CoA formed in the organism. The principal reactions forming acetyl CoA in the animal organism are shown in Fig. 1. To simplify the drawing the intermediary stages are omitted, and only in a few reactions closely connected with CoA a circle indicates the intermediary stages.

It is not possible in the present brief survey to describe the intermediary stages, and reference is only made to the numerous reports published on the subject (*e. g.*, Martius and Lynen 1950, Barker 1952, Lang 1952, Novelli 1953, Ochoa 1952, 1954, Lynen 1954, 1957, Krebs 1950, 1954, Krebs and Kornberg 1957, Lynen and Decker 1957, Wieland 1958).

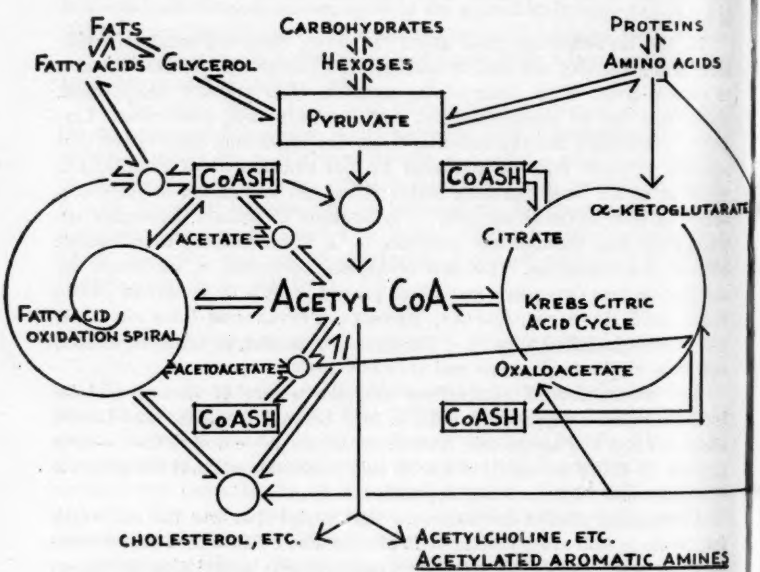


Fig. 1. — Formation of acetyl coenzyme A. (Modified from Novelli 1953.) CoASH = coenzyme A; Acetyl CoA = acetyl coenzyme A.

PRESENT INVESTIGATION

SUBJECTS OF INVESTIGATION

When the present investigation was being planned, it was known from experiments described in the literature that definite differences are demonstrable between male and female animals in the capacity to acetylate aromatic amines. Female rabbits show a more powerful acetylation of sulphanilamide than males *in vivo* and their liver pulp is more active also *in vitro*. Among rats, on the contrary, the males are capable of acetylating both *p*-aminobenzoic acid and sulphanilamide *in vivo* more powerfully than the females. It has further been shown that testosterone has a stimulating action on the aromatic amine-acetylating enzyme prepared from pigeon liver, whereas this effect is not demonstrable with oestrogen.

To better elucidate the effect of the sex glands on the acetylation of aromatic amines, a plan of study was made to investigate the acetylating power of male and female rats and the effect of castration on this power. The investigation was divided into three sections, *i.e.*, experiments *in vivo* and *in vitro*, and determinations of coenzyme A content.

Experiments in vivo were made to study the following subjects:

— The effect of various factors on the capacity of male and female rats to acetylate aromatic amines;

— The effect of castration of male and female rats on their acetylating activity for aromatic amines.

Experiments in vitro were made to study the following:

— The optimum conditions for the acetylating activity of the rat liver for aromatic amines and the effect of various additional factors and inhibitors on this activity;

— The possible occurrence of the acetylating activity for aromatic amines in other rat tissues;

— The effect of sex and castration on the acetylation of aromatic amines in rat liver;

— In the event of acetylating activity in other rat tissues, the effect of sex and castration on also this activity.

Coenzyme A determinations:

— Analyses were made to determine the coenzyme A content of the livers of non-castrated and castrated male and female rats.

EXPERIMENTAL TECHNIQUES

IN VIVO EXPERIMENTS

Rats of the Wistar strain were used in the experiments. Healthy animals of the same age were divided into groups according to weight and were marked by toe amputation. All the rats were first subjected *in vivo* to the *p*-aminobenzoic acid test to determine their acetylating power. On the basis of these results the males with the highest and the females with the lowest acetylating power were selected for castration.

The castration was carried out under light ether anaesthesia. The operation on males was performed by a short incision in the scrotum, from which the spermatic cord, testicle and epididymis were removed *en bloc* after ligation with catgut. The cavity was sprinkled abundantly with powdered potassium penicillin-streptomycin. The wound was carefully closed with a continuous catgut suture. For castration of the females a short median incision was made dorsally, from which a wound about 3 mm in length was cut laterally on each side into the back muscles. The ovaries were extracted through this wound with tissue forceps, the uterine horn was ligated with catgut, and the ovaries and uterine tubes were removed *en bloc*. Powdered potassium penicillin-streptomycin was sprinkled into the abdominal cavity and under the skin of the back. The wound in the back muscles was closed with a catgut suture and the skin with agraffes. Following the operation the animals were kept in heated sterile cages for one week, at the end of which the agraffes were removed from the females. The animals were used for experiments *in vivo* one month after castration.

Before the *in vivo* experiments the animals fasted overnight. The rats were then weighed and numbered, and the substance under examination was diluted in 5 ml of water and administered into the stomach through a rubber tube. The rats were then placed in individual cages, which were constructed to prevent mixing of the faeces and the urine. The administra-

tion of water by stomach tube to rats in such a large quantity has the tendency to diminish experimental errors, such as retention of urine in the bladder and individual variations in the rate of urine flow. It has further been shown by PITKÄNEN (1956) that after the administration of water to rats in a quantity of 3—7 ml signs of stress are not detected. The urine was collected in most experiments during a period of 4 hours, because during that time the excretion rate of *p*-aminobenzoic acid can be followed, as is shown later in the experimental part of this report. At the end of the experimental period the rats were returned to their feeding boxes, the urine was poured into a graduated cylindre, and the collecting vessel was rinsed with 5 ml of water, which also was poured into the same cylindre. The total volume less 5 ml was noted down as the volume of urine. The diluted urine was stirred carefully and two samples of 0.2 ml each were drawn with a tuberculin syringe and placed into numbered test tubes containing 10 ml of water, with which the syringe was rinsed a number of times. To prevent hydrolysis, these urine samples, diluted about 100 times, were stored in the refrigerator at + 4°C until analysed. Each test series comprised 40 rats. Individual samples and volume measurements were made from each rat in this manner. When the experimental time was 4 hours, 80 rats could be examined daily with this technique.

IN VITRO EXPERIMENTS

Preparations from rat tissues were used in the *in vitro* experiments. About 100 of the rats used in the preliminary experiments were of the Sprague-Dawley strain. The remaining rats and those used in the actual experiments were of Wistar strain, which has been bred in this laboratory for a number of years, is healthy and shows very little individual variation.

The animals were fasted for one night before the experiments, killed with a blow on the head without anaesthesia, and immediately thereafter decapitated to remove most of the blood. At the same time the abdominal cavity was opened, using a wide incision, and flushed with running cold (+ 4 — + 6° C) tap water to rapidly cool the viscera. The cooled intact viscera were quickly removed without injury and were cleaned of connective tissue and, by lightly pressing, of blood under running cold water. They were then kept on ice for 5—10 minutes, dried with filter paper and rapidly weighed. The necessary amount of tissue was then cut from the organs with an accuracy of ± 10 mg.

The weighed tissue pieces were immediately placed into 50 ml Erlenmeyer flasks into which had previously been pipetted the used amount of ice-cold reaction fluid and which were kept on ice.

The majority of the experiments were made with tissue cuttings (KINNUNEN 1946). To prepare the cuttings the section of tissue in the cold fluid in the reaction flasks kept on ice was cut with sharp stainless

steel scissors into small pieces about 1 × 1 × 1 mm in size. In cutting the tissue, care was taken to prevent crushing, so that the supernatant fluid remained transparent. In a few experiments tissue slices were used. These were prepared in a cold-room (+ 2 — + 4°C) with a slicer (Stadie and Riggs 1944), with which uniform slices about 0.5 mm in thickness are rapidly obtained. The slices were transferred with forceps from physiological saline solution to a torsion scale, weighed, and immediately transferred to a reaction flask kept on ice and containing reaction fluid, as above. The volume of reaction fluid used for the cuttings and slices was 5, 10 or 15 ml; using 1, 2 or 3 g of tissue the tissue concentration was 10 or 20 per cent (fresh weight per volume).

The homogenates used in a few experiments, were made from liver prepared in the above manner in a Bühler homogenizer equipped with a cooler, which was filled with ice water. Speed 10 and a homogenizing time of 30 seconds were used. The homogenate was diluted with cold medium, usually to a concentration of 10 per cent (fresh weight per volume), and 10 ml of this dilution was used.

The following suspending media for rat tissues were used for cuttings and slices.

In preliminary investigations: Krebs-Ringer phosphate solution, pH 7.4, was prepared immediately before the experiment from stock solutions stored in the refrigerator, following the instructions of Umbreit, Burris and Stauffer (1949).

In most experiments a mixture of the Krebs-Ringer phosphate solution and 0.1 M phosphate buffer in the ratio 1: 1 was used, since the buffering capacity of the phosphate solution proved to be too low. The phosphate buffer was prepared before each series of experiments by mixing appropriate quantities of 0.1 M secondary sodium phosphate and primary potassium phosphate solutions to get pH 7.4. This medium was termed the ***standard medium***.

In studying the effect of ions 0.154 M sodium chloride and the above mentioned phosphate buffer were mixed 1: 1.

For determination of the optimum pH and for homogenates was used 0.1 M potassium phosphate buffer, which was prepared by mixing appropriate amounts of 0.1 M primary and secondary potassium phosphate solutions to the desired pH.

For study of the effect of phosphate, tris buffer, 2-amino-2-hydroxymethylpropane-1:3-diol, »Sigma 7—9» (Sigma Chemical Co.) was used in concentration 0.05 M in aqueous solution, pH 7.4.

The pH values of the buffers were checked repeatedly with a »Radiometer» pH meter.

In all the experiments on the effect of sex a special reaction mixture was used. The composition of this fluid is described in connection with the results of these experiments (p. 73).

The solutions of SUBSTRATES and OTHER ADDITIONS were as follows:

AVERTIN. — This was Brometholum (Bayer), tribromoethanol used in 0.02 M aqueous solution.

BENZOATE. — Sodium benzoate, 1.0 M, in aqueous solution.

COENZYME A. — Because of the lack of a pure standard, a concentrated »Kochsaft» of rat liver was used. To prepare this, the rat livers were deep frozen to break the cells and then cut with scissors in cold 0.1 M secondary sodium phosphate to form a 50 per cent pulp. The pulp was placed in a 100°C boiling water bath for 10 minutes. After boiling it was cooled and centrifuged for 1 hour at +2°C, using 3,000 r.p.m. The clear centrifugate was taken up and its CoA content was determined by the method of KAPLAN and LIPMANN (1948). A small amount of 0.1 M primary potassium phosphate was added and the centrifugate was diluted with 0.1 M sodium potassium phosphate buffer to a concentration containing 50 units of CoA per ml and adjusted to pH 7.4. The solution was stored in the deep-freeze.

CYSTEINE. — Cysteine hydrochloride, 0.02 M, in aqueous solution, stored in the deep-freeze.

MAGNESIUM. — Magnesium sulphate in 0.154 M aqueous solution.

PARA-AMINOBENZOIC ACID. — Acidum aminobenzoicum para (Schering-Kahlbaum A.G., Berlin), a 0.02 M solution in 0.02 M potassium bicarbonate.

SODIUM ACETATE. — 1.0 M, in aqueous solution.

SULPHANILAMIDE. — A solution of 0.02 M in 0.02 M potassium bicarbonate.

Unless otherwise stated, the reagents used were *pro analysi* products of Merck, or of a corresponding quality.

The substrate and other additions were added to the suspending medium in volumes to give the concentration required in each experiment, usually 10 or 15 ml.

Oxygen was used as the gas phase. When a complete test series was ready, all the flasks were transferred at the same time from the ice to a stand in which about 1000 ml of oxygen passed through each flask. Ordinary tank oxygen was used. The flasks were then closed immediately with rubber stoppers and the entire series of flasks was transferred to a water bath thermostat equipped with a mechanical shaker. In most experiments the flasks were incubated at +37°C for 4 hours.

Samples were taken after the incubation. The duplicate samples, usually 1 ml, were transferred to centrifuge tubes containing 4 ml of 5 per cent trichloroacetic acid for the precipitation of proteins. The tubes were kept in the refrigerator for about 4 hours, after which they were centrifuged. The clear supernatant obtained was used for the analyses.

COENZYME A DETERMINATIONS

The rat was killed and the liver removed and cooled as in the *in vitro* technique. From the cooled liver a piece of tissue weighing 1 g (with an

accuracy of ± 10 mg) was cut into a dry, numbered, 50 ml beaker glass kept on ice. When a complete series had been prepared in this manner the liver samples were placed in the refrigerator at —18°C. On the following day the frozen liver tissue was throughly minced into 4 ml of distilled water. The test tubes containing these 20 per cent (W/W) suspensions were placed in a boiling water bath for 5 minutes, cooled in running water and centrifuged. The clear supernatant fluid was stored in the deep-freeze until tested. Since the final volume of the 20 per cent liver tissue suspension was greater than 4 ml but less than 5 ml, it was considered that 1 ml of the supernatant fluid was equivalent, on the average, to 225 mg of fresh liver, and the CoA contents of the liver samples were calculated on this basis. The values thus obtained are relative only, but since they were obtained and calculated in an identical manner they may be regarded as fully comparable.

The acetone-dried, powdered pigeon liver needed in the experiments was prepared as described by TABOR (1955).

The CoA determinations were made by the method developed by KAPLAN and LIPMANN (1948) and modified by NOVELLI (1956, 1957). The standard CoA used in each series of determinations was a dilution series from the stock solution of the concentrated rat liver Kochsaft, which was stored in the deep-freeze in 3 ml batches. The dilutions of the standard were equivalent to 0.3, 0.7, 1.0, 1.3, 1.7, 2.0, 2.7, 3.3 and 6.0 units of CoA. From the centrifugates under examination six dilutions were prepared for double determinations, and the CoA content was calculated from three dilutions falling on the linear portion of the standard curve. One experimental series consisting of the samples from a male, castrated male, female and castrated female rat was prepared and analysed at a time.

METHODS OF ANALYSIS

Most of the chemical methods of analysis of *p*-aminobenzoic acid (PAB) and sulphanilamide are based on the fact that, being primary aromatic amines, these substances may be diazotized with nitrous acid, and another aromatic substance may be coupled with the diazo compound so formed. The coloured compound formed in this way may then be determined spectrophotometrically.

Among methods of this kind may be mentioned the quantitative determinations of MARSHALL (1937), MARSHALL, EMERSON and CUTTING (1937), MARSHALL and LICHTFIELD (1938), HECHT (1938), BRATTON and MARSHALL (1939) and ECHERT (1943), in all of which some naphthyl amine derivative is employed for coupling. For the naphthylamine derivative thymol was substituted by KIMMIG (1943) and thiamine by KIRCH and BERGEIM (1943).

When PAB in an acetic acid solution reacts with an 1 per cent solution

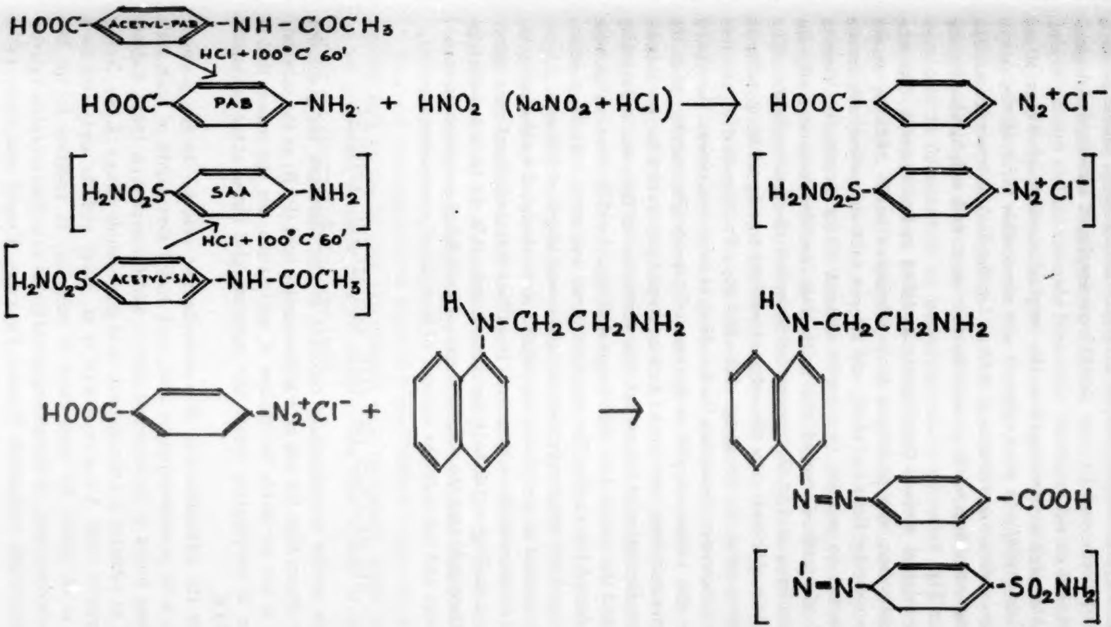


Fig. 2. — Determination of aromatic amines by method of BRATTON and MARSHALL (1939): Sample is treated with nitrous acid to diazotize any amine free at the amino group; diazotized amine is then coupled with N-(1-naphthyl)-ethylenediamine to form a red color. Acetylated amines are diazotizable only after acid hydrolysis. PAB = *p*-aminobenzoic acid. SAA = sulphanilamide.

of *p*-dimethylaminobenzaldehyde in acetic acid a deep yellow colour is formed. TAUBER and LAUFER (1941) have developed a method of determination based on this reaction.

In the present investigation the original method of BRATTON and MARSHALL (1939) was used, since it was serviceable for both the *in vivo* and the *in vitro* experiments as well as for the CoA determinations. The reactions occurring with *p*-aminobenzoic acid and sulphanilamide are shown in Fig. 2.

The original method was slightly modified in two respects. The time of diazotization was prolonged from 3 minutes to 15 minutes, as was already done by ECHERT (1943), and 0.5 per cent ammonium sulphamate was allowed to act for 15 minutes instead of for 2 minutes. Tests to control the method showed that with these modifications and with the concentrations used in the present investigation the results did not differ from those obtained with the original method in respect to quantity or absorption curve in the range 420—640 mμ. Prolongation of the time intervals, however, facilitates the handling of large test series. The optical density was measured with a Beckman B spectrophotometer when the test tubes had been kept in the dark at room temperature for 4—20 hours after the formation of the colour. Gas formation is then not a disturbing factor and the colour does not change during at least 36 hours if the tubes are protected from light. The standard curve was made both for *p*-aminobenzoic acid and sulphanilamide using the wavelength of 530 mμ.

The amount of acetylated conjugates was calculated by deducting the amount obtained without hydrolysis from the amount obtained after hydrolysis in a boiling water bath for 60 minutes. It is to be noted that the result then indicates the total amount of acetylated *p*-aminobenzoic acid conjugates and not only the amount of acetylated *p*-aminobenzoic acid.

SELECTION OF THE AROMATIC AMINES

From earlier investigations reported in the literature the conclusion may be drawn that the use of *p*-aminobenzoic acid (PAB) as the substrate *in vivo* is not advisable for studies of acetylation, for the reason that in addition to conjugating acetyl the organism also forms other conjugates from PAB.

After the administration of *p*-aminobenzoic acid to the rat, *p*-aminobenzoic acid, *p*-aminohippuric acid, and acetyl derivatives of both acids have been found to be excreted (RIGGS and CHRISTENSEN 1951). Rabbits excrete in addition *p*-aminobenzoic acid glucuronide (BRAY, LAKE, NEALE and THORPE 1948, VENKATARAMAN *et al.* 1950) and its acetylated form (BRAY *et al.* 1948). In man there is excreted, in addition to the free *p*-aminobenzoic acid, all three conjugated forms as well as acetylated glycine and glucuronide conjugates (TABOR, FREEMAN, BAILEY and SMITH 1951).

It has been suggested that a competitive equilibrium exists between acetyl and glycine conjugations (Riggs and Christensen 1951) and between acetyl and glucuronide formation (Venkataraman *et al.* 1950).

Glycine conjugation does not occur when sulphanilamide is used. Opinions also differ with respect to the formation of glucuronide. In the opinion of some investigators there is no excretion of this substance (Gilligan and Beck 1945, Scudi and Robinson 1941), while others have been able to demonstrate an increased urinary excretion of glucuronide after the administration of sulphanilamide (Sammons, Shelswell and Williams 1941, Martin and Rennebaum 1943, Williams 1943). The excretion of »ethereal sulphates» in the urine has also been found to be increased (Shelswell and Williams 1940) and 3-hydroxylsulphanilamide has also been demonstrated (Williams 1947) after the administration of sulphanilamide. It is furthermore known that sulphanilamide has a destructive action on intestinal bacterial flora in rats, resulting in vitamin B deficiency. Even when the diet contains adequate amounts of thiamine, riboflavin, nicotinic acid amide, adermine and pantothenic acid, definite deficiency states will develop in rats given sulphonamides (Mackenzie, Mackenzie and McCollum 1941, Doft, Ashburn and Sebrell 1942). This is due to the inhibition of folic acid synthesis in the intestinal bacteria by sulphonamide (Welch and Wright 1943).

PAB is not destructive to intestinal bacteria and is less toxic than sulphanilamide. Since it is known that the acetylations of PAB and sulphanilamide in the rat *in vivo* do not differ greatly (Fishman and Cohn 1943) it was considered advisable to choose PAB as the substrate in the *in vivo* experiments and, for the sake of uniformity, also in the *in vitro* experiments, however using also sulphanilamide as a second substrate in the *in vitro* experiments.

To avoid toxic reactions in the rats it was decided to give the *p*-aminobenzoic acid in the *in vivo* experiments in a maximum single oral dose of 100 μmoles/kg of body weight, which is less than 1/4000 of the LD_{50} for the rat (Scott and Robbins 1942).

RESULTS

ACETYLATION OF *p*-AMINOBENZOIC ACID (PAB) *IN VIVO* IN THE RAT

In the *in vivo* experiments, which are presented in this section, the effects of various factors on the conjugation of *p*-aminobenzoic acid to the amino group was studied in male and female rats of the Wistar strain.

The acetylation of PAB was first studied in male and female rats of the same weight. Experiments were then made to determine the effect of the dosage of PAB, experimental time, body weight, repeated PAB tests, and administration of benzoate on the ability of male and female rats to acetylate PAB. The effect of castration of both sexes on the PAB acetylating power was finally investigated.

ACETYL-PAB FORMATION IN MALE AND FEMALE RATS

Of sexually mature rats of the same age, males usually have a higher body weight than females. If a study is made of the ability of rats of the same age to acetylate the same dose of PAB without consideration to body weight the results may naturally be expected to show that the males, having a greater weight, will acetylate a larger proportion of this dose.

To prevent such a weight difference, a large number of male and female rats of the same age were given a standard diet, the amount of which was decreased for the males and increased for the females. Rats weighing 160—180 g were selected by weight, and from these animals 20 male and 20 female rats were picked out to give a mean body weight of 170 g for each sex. The total weight of the 20 males was 8 g higher than that of the females, the mean weight per animal thus being 170.4 g for the males and 170.0 g for the females. Since this difference was less than 0.3 per cent, the use of these groups was considered satisfactory.

Fig. 3 shows the results of PAB test made on all the rats at the same time on the same day. The ordinate on the left shows the percentage of excreted *p*-aminobenzoic acid that was conjugated to the amino group. The ordinate on the right shows the amount of PAB (in μmoles) conjugated to the amino group and excreted during the experimental time. Column 1 gives the percentage of

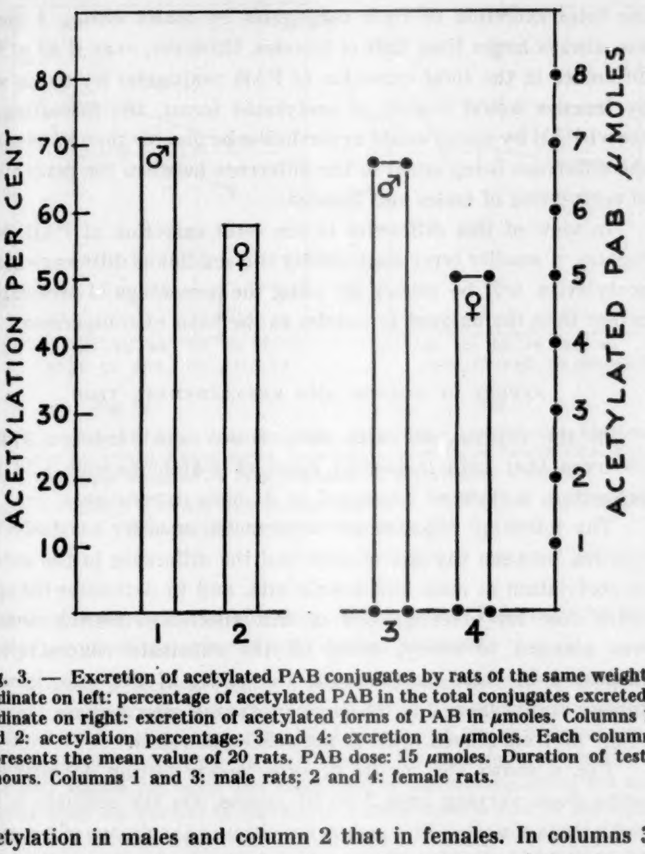


Fig. 3. — Excretion of acetylated PAB conjugates by rats of the same weight. Ordinate on left: percentage of acetylated PAB in the total conjugates excreted. Ordinate on right: excretion of acetylated forms of PAB in µmoles. Columns 1 and 2: acetylation percentage; 3 and 4: excretion in µmoles. Each column represents the mean value of 20 rats. PAB dose: 15 µmoles. Duration of test: 4 hours. Columns 1 and 3: male rats; 2 and 4: female rats.

acetylation in males and column 2 that in females. In columns 3 and 4 are shown the excretion, in µmoles, of the acetylated conjugates by male and female rats in the same experiment. It is seen that the relative difference in the acetylation in males and females was greater when the excretion of acetylated forms in µmoles was used as criterion. The dose in this experiment was 15 µmoles of *p*-aminobenzoic acid and the time of experiment was 4 hours, during which the males excreted 9.4 µmoles and the females 8.8 µmoles of all *p*-aminobenzoic acid conjugates.

In also all the experiments conducted later it was found that

the total excretion of PAB conjugates by males during 4 hours was always larger than that of females. However, even if all of the difference in the total excretion of PAB conjugates by males and by females would consist of acetylated forms, the formation of acetyl-PAB by males would nevertheless be greater than in females, the difference being equal to the difference between the percentage of acetylation of males and females.

In view of this difference in the total excretion of PAB conjugates, a smaller error augmenting the sex-linked difference in the acetylation will be caused by using the percentage of acetylation rather than the amount in μmoles as the basis of comparison.

EFFECT OF DOSAGE AND EXPERIMENTAL TIME

In the experiments with rats, RIGGS and HEGSTED (1949) observed that with increasing doses of *p*-aminobenzoic acid the proportion acetylated decreased in 24-hour experiments.

The following experiments were made in order to study the relation between the size of dose and the difference in the extent of acetylation in male and female rats, and to determine the optimum dose for investigations of this difference. Furthermore it was planned to study, using all the substrate concentrations employed in these experiments, the effect of the experimental times varying from 2 to 24 hours by taking intermediate samples of 0.1 ml from stirred urine in the collection vessel.

Fig. 4 shows the results of experiments lasting 4 hours and using doses varying from 3 to 60 μmoles. On the ordinate is the percentage of acetylation and on the abscissa the dosage of *p*-aminobenzoic acid in μmoles. The steepest decline in the extent of acetylation was seen when the dose was increased in the range 3—15 μmoles. The decrease thereafter was less steep, being about 10 per cent in the range 15—60 μmoles. The difference between the sexes increased up to 15 μmoles, after which it seemed to decrease slightly.

The corresponding results from the 24-hour urine of the same rats are given in Fig. 5, in which the ordinate and abscissa are same as those in Fig. 4. The most marked difference in these results, as compared to those in Fig. 4, was the decline in the

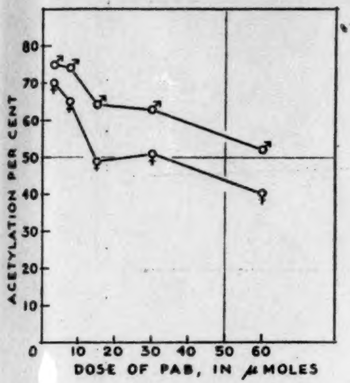


Fig. 4.

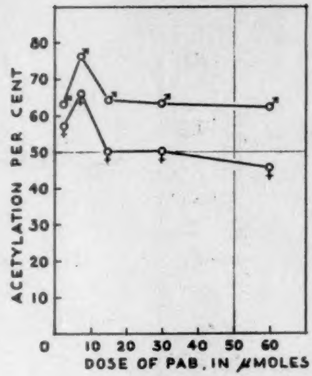


Fig. 5.

Fig. 4. — Effect of PAB dose. Ordinate: percentage of acetylation. Abscissa: PAB dose in µmoles. Upper curve: male rats. Lower curve: female rats. Each dot is the mean value of 20 rats. Duration of experiment: 4 hours.

Fig. 5. — Effect of PAB dose. Same rats as in Fig. 4, but experimental time 24 hours. Ordinate and abscissa same as in Fig. 4.

extent of acetylation of the dose of 3 µmoles. This may be due to hydrolysis of the urine sample during 24 hours.

This phenomenon was studied a number of times by keeping the urine in the collection vessels in the animal stable. Decomposition was not observed until 8—10 hours after the beginning of the experiment, and it continued from the 8th to the 24th hour at the same rate, varying from 0 to 0.03 µmoles per hour. The magnitude of the decomposition did not depend upon the amount of acetylated *p*-aminobenzoic acid in the urine when the latter was in the range 2—20 µmoles. The conclusion may therefore be drawn that the cause probably was not a chemical hydrolysis but rather a possible enzymic reaction produced by bacteria which had entered the urine. Toluene or other preservatives were not used during collection of the urine.

It is further noted from Fig. 5 that in the range 15—60 µmoles the extent of acetylation during 24 hours declined less than it did in Fig. 4, whereas the difference between the sexes was greater.

Since the decrease in the percentage of acetylation is more evident in the 4-hour test than in the 24-hour test, as seen from

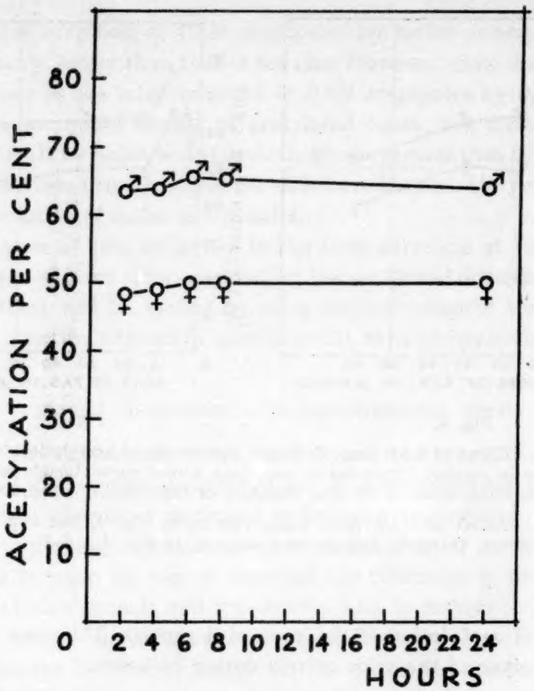


Fig. 6. — Effect of experimental time. Ordinate: percentage of acetylation. Abscissa: experimental time in hours. Each dot is the mean of 20 rats. Upper curve: males. Lower curve: females. PAB dose: 15 μmoles.

a comparison of these two charts, an experimental period of 4 hours and a dose of 15 μmoles of PAB were selected for the continued experiments. The dose of 15 μmoles gives a clear-cut difference between the sexes and the percentage of acetylation undergoes hardly any change during 2—24 hours, as is seen in Fig. 6. In this chart the percentage of acetylation is shown on the ordinate and the experimental time on the abscissa. With larger doses the sex difference increased as the time of experiment was prolonged, and, therefore, when such doses are used for the study of sex-linked differences, the use of experimental times over 4 hours is recommended.

In their investigations RIGGS and HEGSTED (1951) have elucidated the relationship between the body weight of the animal, the dosage, and the total amount of acetylated derivatives excreted. According to their investigations, the amount of acetylated *p*-aminobenzoic acid formed per kg of body weight is directly proportional to the size of the dose of *p*-aminobenzoic acid per kg.

In the present investigation all the experiments were carried out using the same dosage of 15 μmoles for all the animals, for in

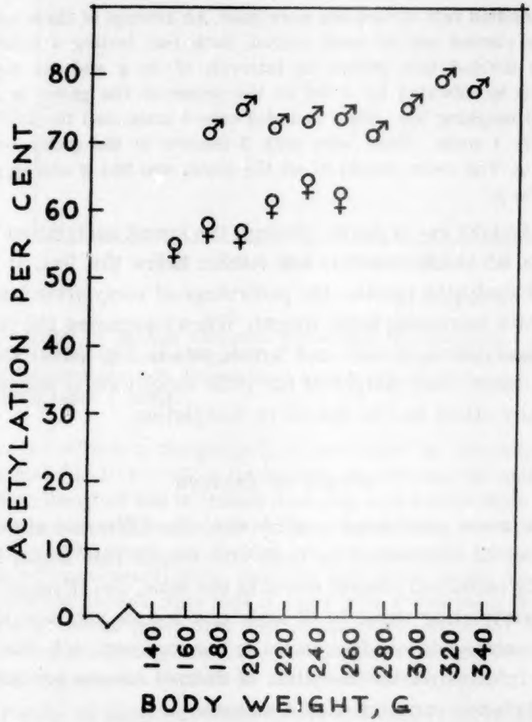


Fig. 7. — Effect of body weight of animals. Ordinate: percentage of acetylation. Abscissa: body weight of rats in g. Upper group: means of male rats. Lower group: means of female rats. PAB dose: 15 μmoles. Experimental time: 4 hours.

large experimental series it was technically difficult always to change the PAB dosage according to the body weight of each animal. The body weights, however, ranged from 145 to 330 g, and the doses of PAB used accordingly ranged inversely from 104 to 45 μmoles per kg of body weight.

After 1000 tolerance tests the effect of body weight on the percentage of acetylation was calculated. The results are shown in Fig. 7, the percentage of acetylated PAB being given on the ordinate and the body weight of the experimental animals on the abscissa.

Two hundred rats of each sex were used. An average of three tolerance tests were carried out on each animal, each test lasting 4 hours. The rats were divided into groups at intervals of 20 g and the mean for each group is indicated by a dot at the centre of the group in Fig. 7. The group weighing 300—320 g included only 4 males and the 320—340 g group only 1 male. There were only 3 females in the group weighing 240—260 g. The mean weight of all the males was 252 g and of all the females 184 g.

If a straight line is drawn through the lowest acetylation values for males, all the female rats will remain below this line. It is also observed that with females the percentage of acetylation increased clearly with increasing body weight. When comparing the percentages of acetylation in male and female rats in Fig. 7 it is also seen that the mean body weight of the male experimental animals had hardly any effect on the extent of acetylation.

EFFECT OF FASTING

Since it was considered possible that the difference observed in the extent of acetylation in male and female rats might be due to readily mobilized acetate stores in the male, the effect of fasting on the acetylating capacity of male and female rats was studied. As the control rats had free access to the standard diet, this should provide information on the effect of dietary acetate precursors on the acetylation capacity of the animals.

In preliminary experiments, 80 male and 80 female rats were selected to form four groups of each sex containing 20 rats. All groups of the same sex had the same mean acetylating power in percentages. The mean body

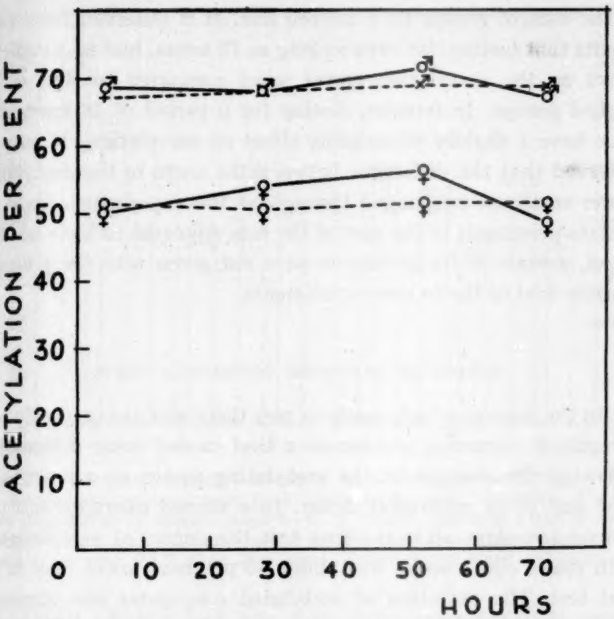


Fig. 8. — Effect of fasting. Ordinate: percentage of acetylation. Abscissa: duration of fasting in hours. Upper line: male rats. Lower line: female rats. Solid lines: experimental rats. Broken lines: control rats. PAB dose: 15 μmoles. Experimental time: 4 hours.

weights of the animals in the groups so formed varied in the range 220—260 g for males and 160—172 g for females. In selecting the animals for these groups the effort was to include male rats with a lower than average acetylating power and female rats with a higher than average power. Each group of 20 animals was divided into two subgroups, one of which served as the experimental group receiving only water during the fasting period, when the animals were kept in cages with a wire mesh floor. The corresponding control group had free access to the standard diet and water before the tolerance test. The test period, excluding the time of fasting, was 4 hours and the dose 15 μmoles of PAB.

The results of these experiments are shown in Fig. 8, in which the ordinate is the percentage of acetylation and the abscissa the duration of fasting at the end of the experiment. The mean values of the experimental groups are connected by a solid line and those

of the control groups by a broken line. It is observed from these results that fasting, for even as long as 72 hours, had no significant effect on the acetylation power when compared to that of the control groups. In females, fasting for a period of 48 hours may even have a slightly stimulating effect on acetylation. It may be observed that the difference between the sexes in the acetylating power remained unchanged throughout the experiment. Since the acetate precursors in the diet of the rats appeared to have no clear effect, acetate or its precursors were not given with the *p*-aminobenzoic acid in the *in vivo* experiments.

EFFECT OF REPEATED TOLERANCE TESTS

In the tolerance tests made *in vivo* there was unexpectedly seen a regularly occurring phenomenon that caused some difficulty in following the changes in the acetylating power as a function of time and of an additional factor. In a second tolerance test performed two days after the first test the degree of acetylation in both males and females was about 30 per cent lower than in the first test. The excretion of acetylated conjugates also showed a similar decline. A third tolerance test performed two days later gave the same depressed values as the second test.

The results obtained in one of these test series are given in Fig. 9, in which the ordinate on the left shows the percentage of acetylation in column pairs 1—3 and that on the right the excretion of acetylated conjugates in column pairs 4—6, expressed in μmoles. Columns 1 and 4 are the results of the first test, 2 and 5 of the second test, and 3 and 6 of the third test. It is noteworthy that the total excretion of *p*-aminobenzoic acid did not change in the three tests.

When the rats were allowed to rest for one month after such a series of tests, their ability to acetylate regained the original level and a repetition of the phenomenon could be obtained, although in a weaker form. The phenomenon was more pronounced in growing rats and weak in rats weighing over 250 g. In young rats of a similar weight it was more clearly seen in males than in females. The administration of water alone did not have the same effect,

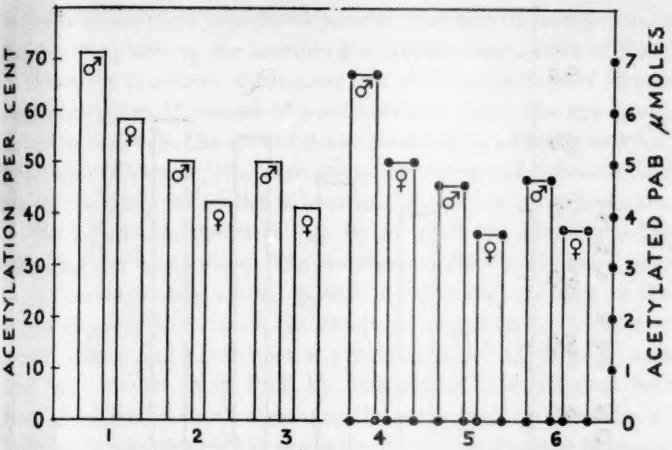


Fig. 9. — Effect of repeated tolerance tests. Ordinate on left: percentage of acetylation in column pairs 1—3. Ordinate on right: excretion of acetylated conjugates in column pairs 4—6, in μmoles. Columns 1 and 4: results of first test; 2 and 5: second test; 3 and 6: third test. Each column is the mean of 20 rats. PAB dose: 15 μmoles. Experimental time: 4 hours.

since PAB administered two days after water gave the original values and only the test made thereafter showed decreased values. For this reason all the experimental groups contained the same number of control animals and the tolerance tests were made on the same day on all the animals in a group in order to subject them to the same number of tolerance tests.

This phenomenon in repeated tolerance tests could not be explained satisfactorily and no reference to it has been found in the literature. Possibly it could be ascribed to activation of the glycine conjugation.

EFFECT OF BENZOATE AND AVERTIN

Riggs and Christensen (1951) observed that the acetyl and glycine conjugations had a reciprocal relationship in the rat in the respect that with a decrease in one conjugation the other increased correspondingly. Since a dose of 15 μmoles of *p*-amino-

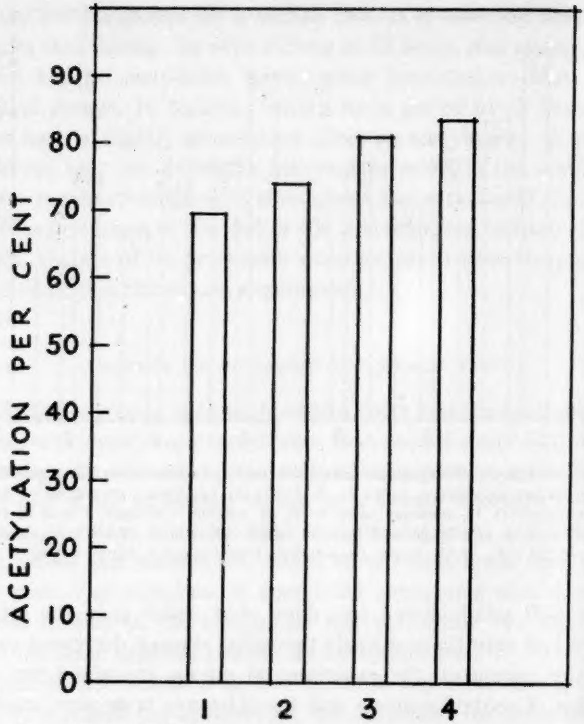


Fig. 10. — **Effect of benzoate and avertin. Ordinate: percentage of acetylation. Each column represents mean of 10 female rats. PAB dose: 15 μmoles. Column 1: controls receiving no additions; 2: addition 30 of μmoles avertin; 3: addition 300 μmoles of benzoate; 4: addition 30 μmoles of avertin and 300 μmoles of benzoate. Experimental time: 4 hours.**

benzoic acid produced in 4 hours a definitely increased excretion of glucuronides in the rat (Luukkainen and Miettinen 1957), it seemed probable that also in the rat not only glycine but also glucuronide would compete with the acetyl group in the conjugation of PAB. It is possible to clearly inhibit the conjugation of PAB and glycine by administration of benzoate along with PAB (Riggs and Christensen 1951). In addition, a large amount of glucuronide conjugates are formed from avertin (tribromoethanol),

and it was therefore considered possible that avertin would also be capable of inhibiting the possible glucuronide conjugation of PAB.

To study the effect of benzoate and avertin, 4 groups of 10 rats each were given 15 μmoles of *p*-aminobenzoic acid. The first group served as controls, the second group received, in addition to PAB, 30 μmoles of avertin, the third group 300 μmoles of benzoate, and the fourth group 30 μmoles of avertin and 300 μmoles of benzoate.

The results are shown in Fig. 10, in which the ordinate is the percentage of acetylation. The numbers under the columns refer to the corresponding animal groups. An increase was seen in the extent of acetylation in all the groups as compared to the control group. Riggs and Christensen (1951) observed in 24-hour tests that the acetylation of PAB by pantothenic acid deficient rats could be increased above normal by the administration of benzoate.

When it was observed in the present investigation that benzoate raised the acetylation percentage in female rats in the 4-hour test it was considered to be of interest to study the effect of benzoate on the sex-linked difference observed in the acetylating power of male and female rats.

The experiment was made on 40 rats divided into 4 groups of 10 rats each, comprising males, castrated males, females and castrated females in separate groups. The experimental time was 4 hours.

The results of this experiment are seen in Fig. 11. The scale for the results expressed in percentages is given on the ordinate on the left, and the ordinate on the right is the μmole scale. The rats first received 15 μmoles of *p*-aminobenzoic acid, the results of this experiment being shown in the dark portion of the columns. The group of columns marked *A* shows the percentage of acetylation in the different groups and that marked *C* the excretion of acetylated forms in μmoles.

Five days after this experiment the same rats received 15 μmoles of *p*-aminobenzoic acid and 300 μmoles of sodium benzoate in 5 ml of water administered through a stomach tube. The full height of the column gives the results of this experiment, and the unshaded portion of the column shows, therefore, the effect of benzoate.

It is seen that both the extent of acetylation and the excretion

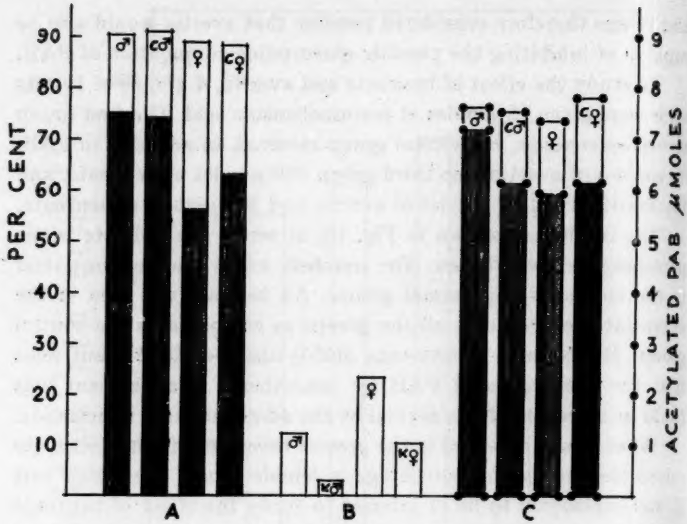


Fig. 11. — Effect of benzoate. Ordinate on left: results expressed in percentages. Ordinate on right: excretion of acetylated conjugates in μmoles. Group *A* columns: percentage of acetylation. *B:* percentage of decrease in the excretion of all forms of PAB after benzoate. *C:* excretion of acetylated forms of PAB in μmoles. Shaded portion of columns: results of tests with 15 μmoles of PAB. Full height of columns: tests with 15 μmoles of PAB and 300 μmoles of benzoate. Unshaded portion of columns: effect of benzoate. Experimental time: 4 hours.

of acetylated forms increased in all the groups after benzoate but that the difference in these respects between the males and the females was insignificant. The *B* group of columns shows the percentage of decrease in the excretion of all forms of PAB after the administration of benzoate. It was observed that through the action of benzoate the *p*-aminobenzoic acid remains in the organism for a greater length of time. Since its excretion therefore is lower, the concentration of the substrate for the enzyme that acetylates PAB in the organism is also higher, at least during an experimental period of 4 hours.

EFFECT OF CASTRATION

The effect of castration on the acetylating ability was studied in 12 complete experimental groups, each comprising 40 rats, *i.e.*,

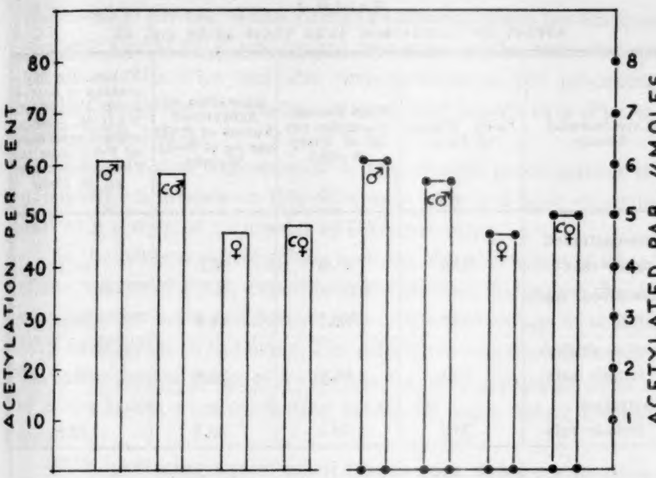


Fig. 12. — Effect of castration. Ordinate on left: percentage of acetylation. Ordinate on right: excretion of acetylated forms of PAB, in μmoles. Columns in both groups from left to right: non-castrated male, castrated male, non-castrated female and castrated female rats. Each column is the mean of 120 tests using 15 μmoles of PAB and an experimental time of 4 hours.

10 males, 10 castrated males, 10 females and 10 castrated females. Two urine samples were taken of every rat, each sample being used for separate determinations of total *p*-aminobenzoic acid and free *p*-aminobenzoic acid. The dose in all cases was 15 μmoles of PAB.

The results of these experiments are shown in Fig. 12. The ordinate on the left shows the percentage of acetylation and that on the right the excretion in μmoles of acetylated PAB conjugates.

Evaluation of the percentage of acetylation in the different groups shows that castration of females increased it slightly over that of non-castrated females, whereas in males castration decreased the extent of acetylation. The excretion in μmoles brings out more clearly the difference in the acetylating power of castrated and non-castrated animals of both sexes.

Table 1 gives the mean body weights of the animals in grammes. As the dose of PAB in all groups was 15 μmoles, the mean dose for each group in μmoles per kilogramme of body weight is shown under

TABLE 1

EFFECT OF CASTRATION. SAME TESTS AS IN FIG. 12

Experimental Group	Body Weight of Rats	PAB Dosage in μmoles per kg of Body Weight	Excretion of Acetylated Forms of PAB per kg of Body Weight	Values for Excretion of Acetylated Forms of PAB in μmoles after Correction of PAB dose to 100 μmoles per kg of Body Weight
Non-castrated male rats ..	228	65.8	26.6	40.5
Castrated male rats	239	62.7	23.6	37.6
Non-castrated female rats	173	86.6	26.6	30.7
Castrated female rats	213	70.5	23.1	32.8

the third heading. The excretion of acetylated PAB conjugates in each group during the 4-hour experiment is listed under the fourth heading in μmoles per kg of body weight. Under the last heading is given the mean excretion of acetylated PAB during 4 hours in μmoles per kg of body weight after correction of the doses in the different groups to 100 μmoles of PAB per kg of body weight, which makes the groups comparable (Riggs and Hegsted 1951). This permits the observations that male rats excreted considerably more acetylated PAB than the females and that the castration of males decreased the excretion only by 7.4 per cent, whereas castration increased the excretion of females by 9.1 per cent. The values under the last heading will be used later in this investigation for comparison with the quantity of formed acetylated *p*-aminobenzoic acid in the *in vivo* and the *in vitro* experiments (pp. 77—79).

DISCUSSION OF THE *IN VIVO* EXPERIMENTS

Dumm and Ralli (1951) demonstrated that the percentage of acetylated *p*-aminobenzoic acid conjugates present in the urine after *p*-aminobenzoic acid administration was greater in male than in female rats in 24-hour experiments using a dose of 1 mg

(7.3 μmoles) per rat. When 70 mg of sulphanilamide per kilogramme of body weight (406 μmoles/kg) was used as the aromatic amine, the same difference was also demonstrable in the percentage of urinary acetylated conjugates in male and female rats (Franz and Lata 1953, 1957).

In the *in vivo* experiments of the present investigation it was possible to demonstrate this difference also in 4-hour experiments and with a dose of 15 μmoles of PAB per rat.

In the present investigation a study was also made of the effect of the dosage of PAB, experimental time and fasting on the difference between male and female rats in the percentage of acetylated PAB conjugates in the urine. The difference was demonstrable over the entire dosage range of 3—60 μmoles, during experimental times of 2—24 hours, or after fasting for 0—72 hours before the experiment.

With increasing age of adult female rats, using as measure the increasing body weight, the difference between the sexes decreased. Since also the activity of the female sex glands is depressed with age, it makes one suspect that the female sex glands are at least in part responsible for the difference in the percentage of acetylated PAB conjugates excreted in the urine by male and female rats. This interpretation was supported by the results obtained after the castration of female rats. In 4-hour experiments the castrated female rats showed a higher excretion of acetylated PAB conjugates than the non-castrated female rats. The decrease seen after the castration of male rats appears to indicate that the male sex glands also play a part in the difference between the sexes in the acetylation of PAB. However, Franz and Lata (1957) and Fishkin and Lata (1957, 1958), in investigations published while the present work was in progress, were unable to demonstrate any change in the sulphanilamide acetylation after the castration of adult male rats when the percentage of urinary acetylated conjugates in 24-hour tests was used as criterion. On the other hand, their observation that the administration of oestrogens to males reduced their acetylating capacity, and the administration of testosterone propionate to female animals increased this capacity, supports the results seen after castration in the present work.

The acetylating power was found not to change in either males or females in experiments in which the effect of fasting up to three days was tested with a dose of 15 μmoles of PAB and compared with unfasted control rats. The conclusion may thus be drawn that the oral administration of acetate precursors has probably no effect on the acetylating power of normal rats. Charalampous and Hegsted (1949) have shown that injections of acetate precursors or adenosine triphosphate to normal rats failed to increase acetylation.

In studying the effect of benzoate administration on the acetylation power of male and female rats, the observation — similar to that of Riggs and Christensen (1951) in 24-hour experiments — was made that benzoate increased the urinary excretion of acetylated PAB. In 4-hour experiments, benzoate also decreased to a marked extent the total amount of PAB conjugates excreted in the urine, *i.e.*, benzoate reduced the rate of excretion of PAB. This is readily understandable, since it is known that glycine conjugation increases the excretion of PAB, whereas the excretion of unconjugated PAB is very small (Beyer, Tillson, Russo, Schuchardt and Pitt 1952).

It is also natural that, in addition to the factors influencing glycine conjugation, all other factors which affect the rate of PAB excretion cause a change in the amount of acetylated PAB conjugates excreted. It is obvious that when PAB is excreted at a rapid rate the organism is unable to conjugate to it many acetyl groups, and when the excretion is slow the organism has time to conjugate more of them. At the same time a high substrate concentration in the organism tends also to stimulate acetylation and thus further raises the amount of the acetylated product.

It seems, indeed, more probable that the increasing effect of benzoate on PAB acetylation is explained by its depressing effect on the rate of PAB excretion, which gives increased opportunity for acetyl conjugation in the organism, rather than by the existence of actual competition between acetyl and glycine conjugations of PAB, as suggested by Riggs and Christensen (1951). A difference in the excretion rate is probably also explained by the difference observed by them between the acetylation of *p*-aminobenzoic acid and *p*-aminohippuric acid.

Nineteen hours after the last of three injections of 10 mg (total dosage about 220 μmoles) of PAB labelled with ^{15}N into male mice weighing 22 g, LUSTIG, GOLDFARB and GERSTL (1944) were able to demonstrate only a trace of radioactivity in the organism. Similar results showing the rapid excretion of PAB in mice were obtained by TABOR *et al.* (1951), who found that immediately after the ingestion of 1 g of PAB per kg of body weight (about 160 μmoles/mouse) the total amount administered was demonstrable in the organism, 4 hours later 50 per cent, and none after 8 hours. In human subjects the excretion of PAB appears to follow similar lines, for STRAUSS *et al.* (1941) reported that after 6 hours only a minute amount of PAB had not been excreted and after 12 hours the excretion was complete in all cases. The results of the 4-hour experiments in the present investigation indicate that the excretion of PAB in the rat occurs at about the same rate as in mouse and man, since 4 hours after the oral administration of 15 μmoles of PAB to the rat 8.3—10.5 μmoles, equivalent to 55—70 per cent of the administered amount, had been excreted.

When an experimental time of 24 hours instead of 4 hours is used, a prolongation of the time of excretion of PAB up to two-fold might occur without being observed, since the urine is only examined 24 hours after the administration of PAB. This considerable source of error in PAB tolerance tests *in vivo* does not show up in the 24-hour experiments.

Although changes in the rate of excretion may be followed in the 4-hour experiment, the effect of the changes on the results cannot be eliminated. Accordingly, accurate results cannot be obtained in the *in vivo* experiments with PAB. It seems probable that the results are, at the most, indicative only of the trend, and may even be misleading. For this reason the results have not been analysed statistically.

ACETYLATION OF AROMATIC AMINES *IN VITRO*

The *in vitro* experiments were made with rat tissue preparations cut with scissors into small sections, or »cuttings» (see p. 19). The results obtained with this technique were more uniform than those

given by other techniques. Similarly it was observed that tissue cuttings converted larger quantities of aromatic amines to non-diazotizable derivatives than tissue slices and homogenates did.

Examination of the results of the *in vivo* experiments described in the foregoing section showed that changes in the acetylating power of rats were small especially after castration. It therefore seemed possible that also in experiments *in vitro* the differences between castrated and non-castrated male and female rats would not be great.

In order that these small differences would not be masked by experimental errors it was necessary to know accurately the conditions under which the conjugation at the amino group of the aromatic amines takes place and what factors influence this conjugation.

The results of the *in vitro* experiments are presented in two parts. The first section describes the experiments on acetylation of *p*-aminobenzoic acid (PAB) and sulphanilamide (SAA) in rat tissue, and in the second section are reported the results of the experiments relating to the effect of sex on PAB acetylation.

ACETYLATION OF *p*-AMINOBENZOIC ACID (PAB) AND SULPHANILAMIDE (SAA) IN RAT TISSUE PREPARATIONS

ACETYLATION OF PAB AND SAA BY LIVER TISSUE

Since little is known concerning the acetylation of aromatic amines *in vitro* in rat tissues it was first necessary to investigate the optimum conditions for this conjugation. The effects of fundamental factors such as preparation of the tissue, gas phase, differences between PAB and SAA as substrate, pH of reaction mixture, incubation time, concentration of the liver tissue in the reaction mixture, and the initial concentration of substrate were studied.

Effect of Type of Tissue Preparations and of the Gas Phase

On the basis of earlier investigations reported in the literature, three types of tissue preparation could be selected for use in the *in vitro* experiments. KLEIN and HARRIS (1938) used rabbit liver

slices, LIPMANN (1945) pigeon liver tissue homogenates and KINNUNEN (1946) rabbit liver »pulp» (similar to cuttings). They observed further that acetylation occurred best under aerobic conditions, being considerably lower under anaerobic conditions.

To find the preparation suitable for rat liver the above mentioned three techniques of preparation were tested in the same experimental series. The possible significance of the use of air or oxygen as the gas phase was tested at the same time.

The arrangement of these experiments was as follows. Livers were taken from male rats that were of the same age and killed at the same time. To diminish the influence of individual variations, the livers (about 100 g) were pooled in a cold room and one slice was cut from all the livers into each incubation flask in the slice experiment series until the flask contained 3 g of tissue. The remaining liver was cut into pieces weighing about 100 mg and thoroughly mixed; 3 g of these pieces were placed in each incubation bottle in the liver cuttings series and cut with scissors as previously explained. The remainder of the liver pieces were homogenized with 0.1 M potassium phosphate buffer, pH 7.4, to a 20 per cent homogenate.

Into the flasks containing slices or cuttings was added 1 μmole of SAA in 5 ml of standard medium per gramme of tissue. Each incubation flask thus contained 3 μmoles of SAA in 15 ml of medium. The 20 per cent homogenates were prepared with 0.1 M potassium phosphate buffer, pH 7.4. Then 5 ml of this homogenate and 5 ml of standard medium were placed in each incubation flask. All the series were incubated at the same time for 4 hours at +37° C. Air and oxygen alternated as the gas phase in every other flask and the pH of all flasks was 7.4.

The results are shown in Fig. 13. The columns give the means of the series and the individual incubation flask results are shown by dots. The unshaded columns represent the series in which oxygen was used as the gas phase, and the shaded columns those series in which air was the gas phase.

Since the homogenates contained one-third of the amount of substrate and tissue used in the other series, the amount of acetylated sulphanilamide in these groups was multiplied by 3 in order to obtain comparable values.

On examination of the results it is observed that in all the series

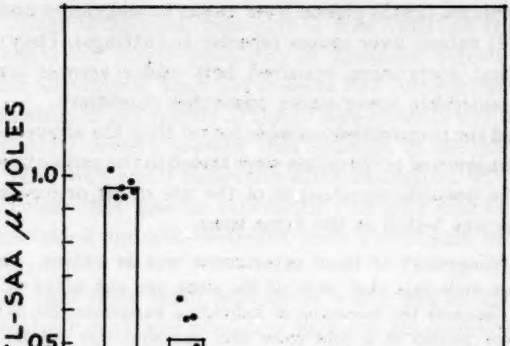


Fig. 13. — Effect of type of tissue preparation and of the gas phase. Ordinate: acetylated SAA formed during 4 hours. Columns are the means of the series; dots show the results of the individual flasks. Unshaded columns: gas phase oxygen. Shaded columns: air. Columns 1 and 4: liver cuttings; 2 and 5: liver slices; 3 and 6: liver homogenates. Substrate 3 μmoles of SAA, amount of liver 3 g. pH 7.4. 37°C.

in which oxygen was used as the gas phase the production of acetylated SAA was greater than in the corresponding series in which air was used for this purpose.

In both the oxygen group and the air group the differences between the results obtained with the different types of preparations were nearly the same. In both groups the largest amount of SAA was acetylated by cuttings. Slices were the next in order. In the homogenate series acetylation was observed in one air group only, and even this was low in quantity.

The dispersion of the results of the individual incubation flasks was smallest in the cuttings series. The greater dispersion in the slice series indicates that the preparation of a uniform quality is difficult with a slice preparation.

To obtain confirmation of these observations a number of test series were made using PAB or SAA as substrate. The results had the same trend as those described above, with the exception that in the homogenate series acetylating activity was observed in only a few flasks incubated in oxygen and in none of the flasks incubated in air.

Since definite differences thus were observed between the various preparations and between the two gas phases, and since tissue cuttings and oxygen were found to give best results, the latter were used in the further experiments. Because homogenates prepared from the same liver as the active tissue cuttings were inactive, the acetylation reaction was considered to be enzymic and bound to the intact cell.

Effect of PAB and SAA as Substrates

The rat organism conjugates to PAB not only the acetyl group (see p. 24) but also glycine, and there probably also occurs glucuronide conjugation. In the case of SAA, on the other hand, acetylation presumably is quantitatively the most important conjugation mechanism.

It is therefore probable that the other conjugation mechanisms may have an inhibitory effect on the acetylation of PAB. In the absence of suitable methods for their determination it is difficult to evaluate their significance. For this reason, preliminary experiments were made to compare the acetylation of PAB with that of SAA, and it was found that when the same molar quantity of both substances was applied the acetylation of both was also the same in quantity.

The technique of the experiments comparing the quantities of acetylated derivatives of PAB and SAA formed under similar conditions was as follows.

Livers of four male rats were cut separately with scissors in beakers into pieces weighing about 100 mg. Every other piece of a rat liver was placed after weighing into incubation flasks containing 15 μmoles of PAB in 15 ml of standard medium. The alternate pieces were similarly weighed and placed in flasks containing SAA and standard medium until

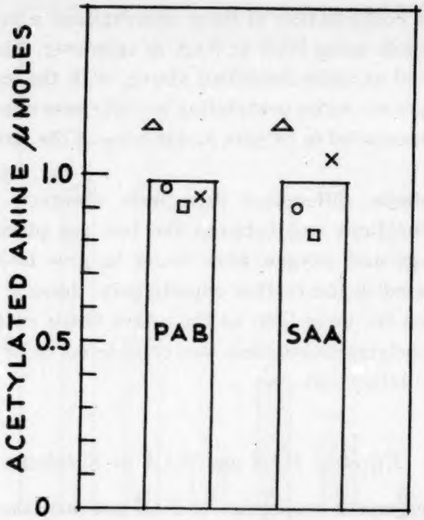


Fig. 14. — Effect of PAB and SAA as substrate. Ordinate: amount of acetylated amines in μmoles formed during 4 hours. △: values obtained with 3 g of the liver cuttings of rat 1; ○: rat 2; □: rat 3; ×: rat 4. Substrate: 3 μmoles of PAB or SAA. pH 7.4. Gas phase: oxygen.

there was 3 g of liver in each flask. The bottles were incubated for 4 hours at 37°C, using oxygen as the gas phase.

The results of the experiment are shown in Fig. 14. The individual values of these four rats were dispersed over a rather wide range. However, the arithmetic mean in the two groups was, by chance, exactly the same.

Although the results of this experiment and the previous ones seemed to indicate that no marked differences occurred in the acetylation of PAB and SAA in liver cuttings, comparisons were made also in the later experiments between the acetylation of PAB and SAA under various test conditions.

Effect of pH

It is an essential characteristic of enzyme reactions that they are dependent on the pH of the reaction mixture. In seeking the opti-

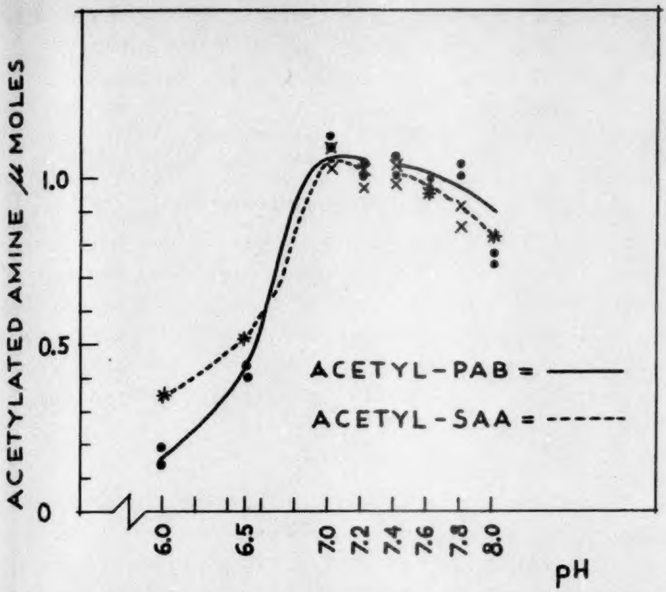


Fig. 15. — Effect of pH. Ordinate: amount of amines acetylated in 4 hours. Abscissa: pH of buffer. Solid line: PAB used as substrate. Broken line: SAA used as substrate. Amount of substrate: 3 μmoles. Amount of liver tissue cuttings: 3 g in 15 ml of potassium phosphate buffer. Gas phase: oxygen. Incubation temperature: 37°C.

mum pH for the acetylation of the tested aromatic amines, determination was made at the same time of the dependence of the acetylation of both PAB and SAA on the pH of the reaction mixture. The range studied was pH 6.0—pH 8.0.

The reaction medium was a mixture of 0.1 M primary and secondary potassium phosphate. To 15 ml of medium were added 15 μmoles of substrate and 3 g of pooled liver from several female rats. The flasks were incubated for 3 hours, using oxygen as the gas phase. The reaction flasks at pH 6.0—7.2 were placed in one water bath and those at pH 7.4—8.0 in another. Incubation of the latter series was started one-half hour later than the first series. Since the series were exceptionally kept on two water baths, the pH curves in Fig. 15 are interrupted between pH 7.2 and pH 7.4.

The results in Fig. 15 show that the optimum pH for both amines was in the range pH 7.0 and 7.8. In this optimum range the pH curves for the two amines showed no noteworthy difference. At low pH levels the acetylation of SAA appears to be somewhat greater than that of PAB.

Effect of Incubation Temperature

All the incubations were carried out in temperature-controlled waterbaths at + 37°C. In preliminary experiments it was observed that the acetylation of SAA and PAB in liver cuttings is dependent on the temperature. At a temperature of 0°C no acetylation was found to occur even during some length of time, and at room temperature (+ 16°C) there was only slight acetylation.

Effect of Incubation Time

In studying an enzymic reaction the usual method of investigation is to observe the rate of reaction by drawing small samples from the reaction flasks at short intervals. The use of oxygen as the gas phase had the disadvantage that when samples were taken during incubation, the oxygen in the flasks tended to escape, which impaired the acetylation yield. When an attempt was made to correct this drawback by conducting more oxygen into the flasks after taking of the samples, this manipulation in a large series required so long a time that the tissues suffered from oxygen deficiency while shaking was interrupted, resulting in a decrease in the rate of reaction. The so-called single-point assay was therefore used. The necessary condition for this assay is that the rate of reaction is constant during the selected experimental time.

To study the effect of the incubation time, liver pieces from several rats were pooled as above described and 3 g of tissue was placed into each reaction flask kept on ice and already containing 3 μmoles of sulphanilamide in 15 ml of standard medium. Gas was conducted into the bottles, which were then closed with rubber stoppers. After gassing for 10 minutes, the zero time flasks were removed from the series and sampled. In the same manner, by stopping the shaker apparatus of the water bath for a moment, flasks were removed at intervals of 1 hour and samples were immediately taken from them.

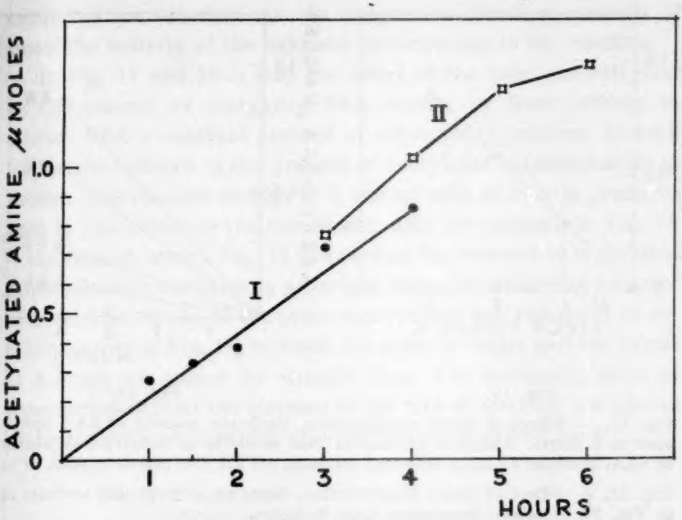
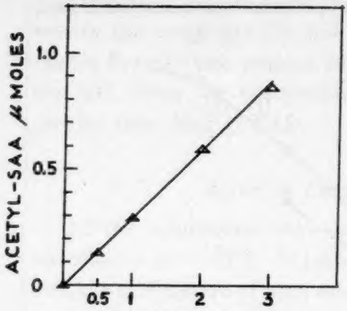


Fig. 16. — Effect of incubation time. Ordinate: amount of amines acetylated. Abscissa: incubation time in hours. Curve I: 3 μmoles of SAA used as substrate in 15 ml of standard medium. Curve II: 3 μmoles of PAB and 300 μmoles of acetate in 15 ml of standard medium. All experiments made with 3 g of liver tissue cuttings at pH 7.4, 37°C, using oxygen as gas phase.

The results of the experiments on the effect of incubation time are shown in Fig. 16 (curve I), in which the ordinate is the amount of acetylated sulphanilamide formed by the liver cuttings, and the abscissa is the time in hours. As is seen from this figure, the 4-hour curve meets fairly well the requirements placed upon it for performance of a single-point assay. Since the reaction unexpectedly continued rectilinearly up to 4 hours, the interval 3—6 hours was later tested a number of times. Curve II has been drawn on basis of the mean values obtained in a test series of this kind. Differing from the first experimental series, the medium in this later experiment was standard medium containing acetate. The rate of reaction shown in curve II was nearly similar to that in curve I up to 5 hours, after which the rise was less steep. On the basis of these experiments and others which gave the same result, 4 hours were chosen as the incubation time during which the maximum amount of



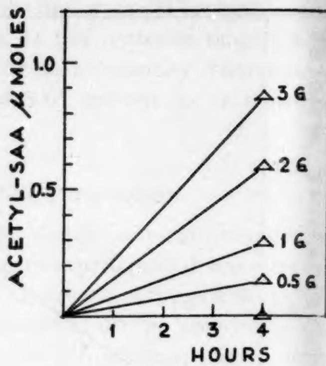


Fig. 17.

Fig. 18.

Fig. 17. — Effect of tissue concentration. Ordinate: amount of SAA acetylated in 4 hours. Abscissa: amount of liver tissue in g. Substrate: 3 μmoles of SAA. Medium: 15 ml of standard medium, pH 7.4. Gas phase: oxygen. 37°C.

Fig. 18. — Effect of tissue concentration. Same experiment and ordinate as in Fig. 17. Abscissa: Incubation time in hours.

aromatic amines was acetylated. With this length of incubation time the slight difference in time in taking samples from the first and last flasks of the series does not represent a noteworthy difference in the incubation time.

Effect of Tissue Concentration

It was already shown in the experiments with homogenates that the studied reaction apparently was an enzymic one, and further that under the reaction condition used it was bound to the intact cell. We know that when the amount of substrate is constant, within certain limits the amount of reaction product of an enzymic reaction depends upon the concentration of the reacting enzyme.

If the measured amount of reaction product were not dependent in at least some range on the amount of tissue, it would be impossible to compare the results obtained with different groups of animals by determining the amount of reaction product formed when the type and weight of tissue and the reaction time and

conditions are identical, *i.e.*, to compare in identical amounts of tissue the activity of the enzymes participating in the reaction.

In Fig. 17 and 18 is seen the effect of the liver concentration on the amount of acetylated SAA formed by liver cuttings in 4 hours with a constant amount of substrate (3 μmoles). In both figures the ordinate is the amount of acetylated sulphanilamide in μmoles. The abscissa in Fig. 17 is the amount of liver in grammes used in the different reaction flasks, and the abscissa in Fig. 18 is the time in hours. Fig. 17 shows that the amount of acetylated sulphanilamide increases as a straight line with increasing amounts of liver. The results of the same experiments are expressed in another manner in Fig. 18, in which the point of origin and the values at 4 hours are joined by straight lines. The increasing slope of these curves depicts the increase in the rate of reaction, the figures at the end of the curves indicating the grammes of liver used.

Effect of Substrate Concentration

The maximum rate of reaction and the saturation point of the enzyme are further features characterizing enzymic reactions. To determine these properties and to find the optimum concentration of substrate a number of experiments were carried out, the most representative of which are shown in Fig. 19 and 20.

The reaction flasks contained 3 g of homogeneous cuttings of male rat livers in 15 ml of standard solution. The substrate was PAB in the experiment shown in Fig. 19 and SAA in that depicted in Fig. 20. The ordinate in both figures is the amount of aromatic amine in μmoles acetylated in 4 hours and the abscissa the substrate concentration in μmoles. The experiments were conducted on different days, but in both cases the following conditions were used: temperature $+37°C$, reaction time 4 hours, pH 7.4, and gas phase oxygen.

It is seen that the saturation point of the enzyme was at 9 μmoles *i.e.*, 3 μmoles per g of liver tissue. The maximum rate of reaction was 1.1 μmoles per 3 g of liver. Furthermore it is observed that the amount of 3 μmoles of substrate per 3 g of liver used in most of the experiments was well over the amount of substrate giving

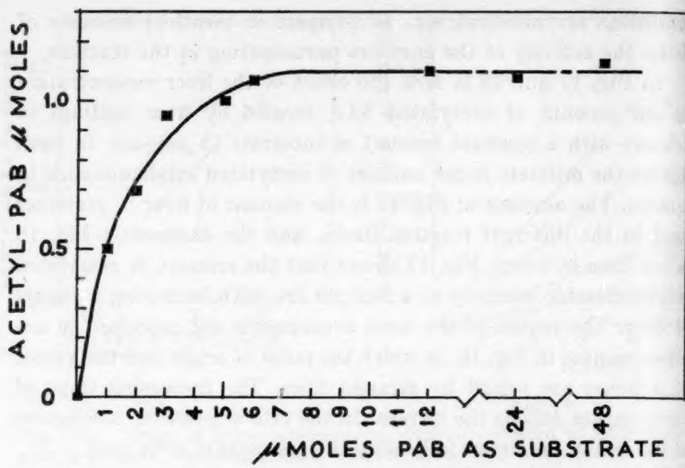


Fig. 19. — Effect of substrate concentration. Ordinate: amount of PAB acetylated in 4 hours. Abscissa: amount of PAB used as substrate, in µmoles. 3 g of liver tissue cuttings and 15 ml of standard medium, pH 7.4, in each incubation flask. Gas phase: oxygen. 37°C.

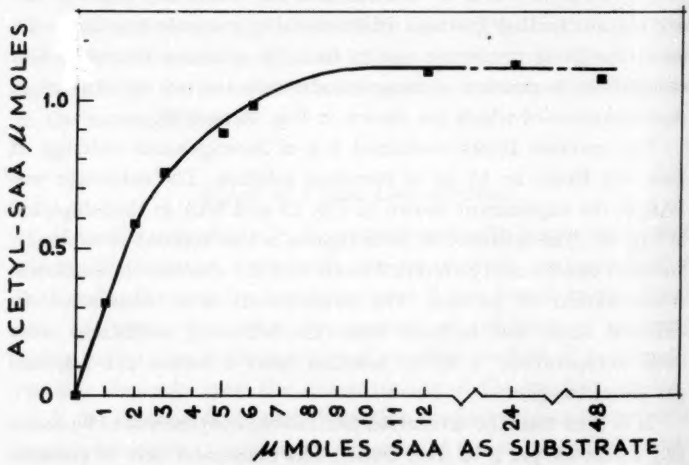


Fig. 20. — Effect of substrate concentration. Ordinate: amount of SAA acetylated in 4 hours. Abscissa: amount of SAA used as substrate, in µmoles. 3 g of liver tissue cuttings and 15 ml of standard medium, pH 7.4, in each incubation flask. Gas phase: oxygen. 37°C.

one-half of the maximum rate of reaction. It was unexpected that the curves for the substrate concentration were so similar for the two aromatic amines used, the enzyme saturation point and the maximum rate of reaction being the same when PAB or SAA was used as substrate.

The possibility was also considered that the level of the curves in the figures was determined by the size of the »acetate store» of the tissue and not by saturation of the enzyme. To study this possibility, additional experiments were made using parallel series prepared with 5 ml of 0.1 M potassium sodium phosphate buffer, pH 7.4, and 100 μmoles of sodium acetate in one series and the same medium without acetate in the other series. The livers, of which only 1 g was used in each reaction flask, were obtained from male rats of the same litter as the rats used in Fig. 19 and 20.

In Fig. 21, in which the results of the experiments are given, the ordinate is, as above, the amount in μmoles of acetylated SAA formed in 4 hours and the abscissa is the amount of substrate in

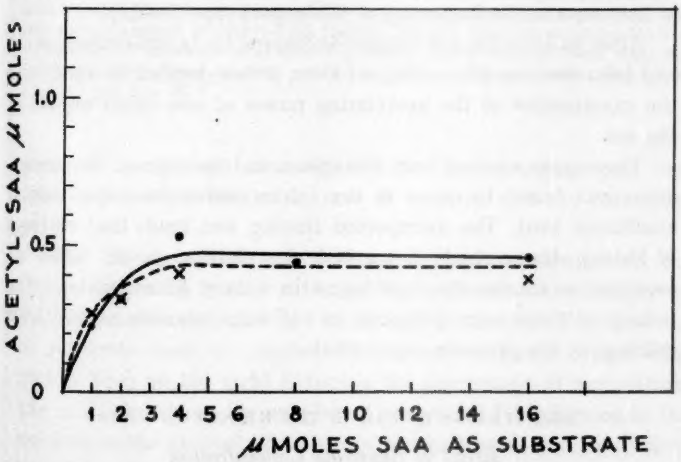


Fig. 21. — Effect of substrate concentration. Ordinate: amount of SAA acetylated in 4 hours. Abscissa: amount of SAA used as substrate, in μmoles. 1 g of liver tissue cuttings in 5 ml of standard medium (solid line curve) or in 5 ml of standard medium containing acetate (broken line curve). Gas phase: oxygen. pH 7.4. Temperature: 37°C.

μmoles. The broken line curve shows the reaction in the experiments with acetate and the solid line curve the reaction without acetate. No noteworthy difference is seen between the two curves.

To confirm the results, random experiments were carried out using large and small amounts of substrate. Differences were uniformly demonstrable only when small amounts were used. A part of these experiments will be described later in this report in connection with other experiments on the effect of acetate.

A comparison of the results in Fig. 21 with those in Fig. 19 and 20 shows that the saturation point per gramme of liver is nearly the same in all cases. The maximum rate of reaction is somewhat greater in Fig. 21 than in the preceding experiments. The addition of acetate therefore appears to have no effect on the saturation point or on the maximum rate of reaction in the liver.

ACETYLATION OF PAB IN OTHER TISSUES

Earlier investigations in this field were reviewed in the survey of literature in the beginning of this report (pp. 13—14).

After an accurate and reliable technique for *in vitro* experiments had been developed by using rat liver, it was decided to undertake the examination of the acetylating power of also other organs of the rat.

The organs selected were the spleen and the kidney. No acetylation was found to occur in the spleen under the experimental conditions used. The unexpected finding was made that cuttings of kidney tissue had a marked acetylating power, while no acetylation whatsoever was found in kidney homogenates. The kidney cuttings were prepared in the same manner as the liver cuttings in the previous experiments.

ACETYLATION OF PAB IN THE KIDNEY *IN VITRO*

Effect of Substrate Concentration

In order to compare the acetylation in the liver *in vitro* with that in the kidney, the latter was examined under the optimum conditions found in the experiments with liver. The most suitable

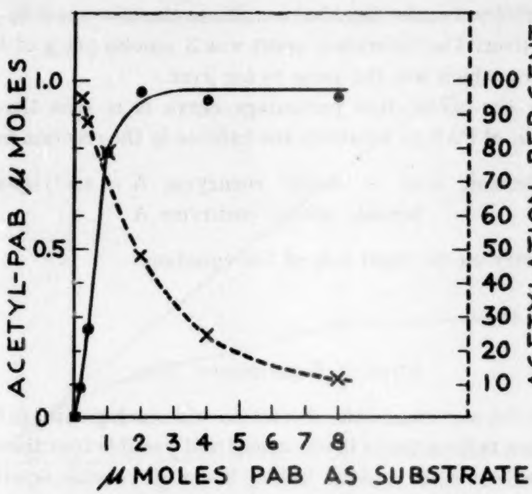


Fig. 22. — Effect of substrate concentration, in the kidney. Ordinate on left: amount of PAB acetylated in 4 hours. Ordinate on right: percentage of acetylation. Abscissa: amount of PAB used as substrate, in µmoles. Solid line curve: amount of acetyl-PAB formed. Broken line curve: acetyl-PAB as percentage of substrate available. 1 g kidney tissue cuttings from male rats in 10 ml of standard medium containing 200 µmoles of acetate. Gas phase: oxygen. pH 7.4. 37°C.

points of comparison of acetylation in the two organs were considered to be the maximum rate of reaction and the saturation point.

Fig. 22 shows the effect of substrate concentration in the experiments with kidney cuttings. The ordinate is the amount of PAB in µmoles acetylated in 4 hours, and the abscissa is the amount of substrate used in µmoles. The second ordinate drawn with broken lines on the right indicates the percentage of acetylation. The solid line shows the amount of acetylated PAB formed in the reaction as the amount of substrate was increased, and the broken line states the acetylated PAB as percentage of the total amount of substrate available.

It is observed that the maximum rate of reaction per 4 hours was 0.96 µmole per gramme of kidney. In the experiments with

liver carried out under identical conditions the rate was 0.45 μmole per g of liver. The saturation point was 3 μmoles per g of kidney in 4 hours, which was the same as for liver.

From the acetylation percentage curve it is seen that with 0.2 μmole of PAB as substrate the balance in the reaction formula

p-aminobenzoic acid + acetyl coenzyme A = acetyl-*p*-aminobenzoic acid + coenzyme A

was entirely on the right side of the equation.

Effect of Experimental Time

Since the maximum rate of reaction was much greater in kidney tissue than in liver tissue it was considered possible that the acetylation also was more rapid in kidney tissue. A possible equilibrium would thus remain unchanged during the remainder of the 4 hours. This lack of information on the course of the reaction is one of the drawbacks associated with the single-point assay.

Experiments were therefore made to study the rate of the reaction and to determine the optimum experimental time for kidney tissue. The results are shown in Fig. 23, in which on the ordinate is the amount in μmoles of PAB acetylated during the time intervals shown by the abscissa.

In series 1 and 2 the reaction flasks contained 1 g of kidney of male rats and 200 μmoles of acetate in 10 ml of standard medium, pH 7.4. The temperature was +37°C and the gas phase oxygen. At the stated intervals flasks were removed from the water bath and sampled.

PAB was used as substrate in the amount of 2 μmoles in series 1 and of 0.5 μmole in series 2. The amount of substrate in series 1 was the same as that used in the later experiments.

On examination of curve 1, it is found to continue almost linearly up to 5 hours, after which the rise is less steep during the sixth hour. However, this finding does not prevent the use of the single-point assay during an incubation time of 4 hours. Curve 2 lags below curve 1 already after one hour.

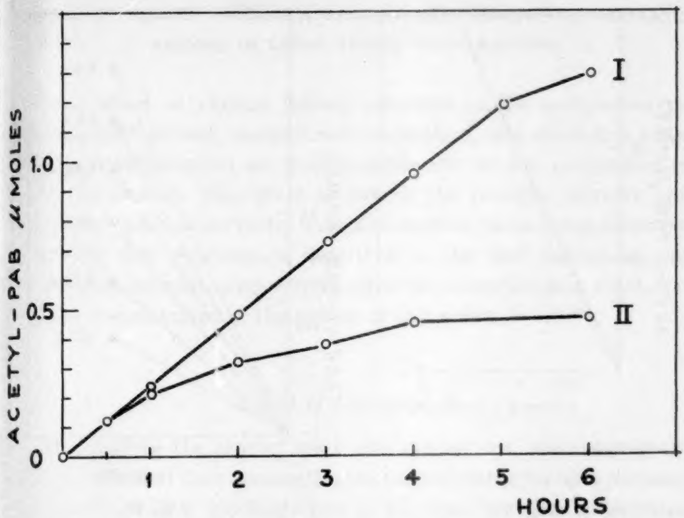
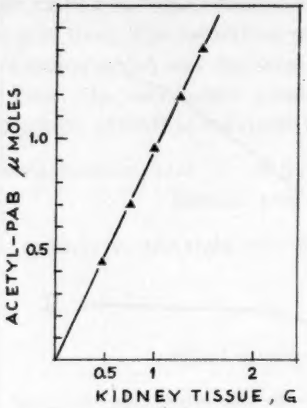


Fig. 23. — Effect of incubation time. Ordinate: amount of PAB acetylated in 4 hours. Abscissa: incubation time in hours. 1 g kidney tissue cuttings from male rats. 200 μmoles of acetate in 10 ml of standard medium, pH 7.4. Temperature: 37°C. Substrate: 2 μmoles of PAB (curve I) and 0.5 μmole of PAB (curve II).

This phenomenon is in a way the same as that seen in the curve for the substrate concentration (Fig. 22) but naturally it is an opposite one, since the rate of reaction decreased as the amount of substrate was reduced. As may be observed from the curves, as much as one-half of the available amount of substrate in reaction II was consumed during the first 1½ hours, whereas in reaction I this occurred only after 4 hours.

Effect of Tissue Concentration

The effect of the concentration of kidney tissue in the reaction mixture on the amount of PAB acetylated in 4 hours is shown in Fig. 24 and 25. The amount of acetylated PAB in μmoles in the ordinate is plotted against the amount of kidney tissue in grammes



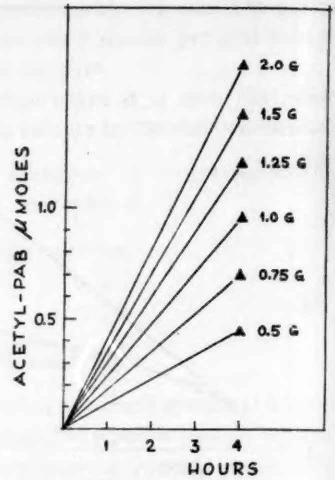


Fig. 24. Fig. 25.

Fig. 24. — Effect of kidney tissue concentration. Ordinate: amount of PAB acetylated in 4 hours. Abscissa: amount of kidney tissue from male rats in g of tissue cuttings. Substrate: 2 µmoles of PAB. 200 µmoles of acetate in 10 ml of standard medium, pH 7.4. Gas phase: oxygen.

Fig. 25. — Effect of kidney tissue concentration. Same experiment and ordinate as in Fig. 24. Abscissa: experimental time in hours.

in the abscissa in Fig. 24 and against the reaction time in hours in the abscissa in Fig. 25. It is observed from Fig. 24 that the amount of PAB acetylated is directly proportionate to the amount of kidney tissue in grammes. In Fig. 25 the results of the same experiment are shown by entering at 4 hours the amounts of acetylated PAB. From the preceding experiments we know that acetylated forms are not yet produced at zero time. It has therefore been possible to connect the point of origin and the 4-hour values by a straight line, whose slope thus indicates the rate of reaction during 4 hours with the amount of kidney tissue used. A greater slope thus indicates a higher rate of reaction. The rate is found to increase uniformly in proportion to the increase in the amount of kidney tissue.

EFFECT OF ADDED FACTORS AFFECTING ACETYLATION OF AROMATIC AMINES IN LIVER TISSUE PREPARATIONS

The effect of various known cofactors in the acetylation reactions, such as CoA, acetate and magnesium, was studied in order to obtain information on their possible rôle in the acetylation of aromatic amines. The effect of ions in the reaction mixture and that of benzoate and avertin were also studied, since it was observed in the *in vivo* experiments described in the first section of this report that benzoate and avertin affect the acetylation of PAB, but no idea was obtained of the nature of this action.

Effect of Coenzyme A

At the time the present work was carried out, necessary quantities of purified CoA standard were not available for this purpose. Standardized liver Kochsaft (see p. 21) was therefore substituted for the pure CoA.

The effect of added Kochsaft was studied in three parallel test series.

In the first series were used 2 μmoles of SAA and 200 μmoles of acetate in 10 ml of standard medium, in the second series 2 μmoles of SAA but no acetate in 10 ml of the medium, and in the third series 0.5 μmole of PAB and 200 μmoles of acetate in 10 ml of medium. The Kochsaft for the experiment, containing about 500 units of CoA per 10 ml, was also divided into three parts, into each of which similar additions of SAA and acetate, SAA only, and PAB and acetate were made. Into the control flasks in all the test series were pipetted 10 ml of the respective medium, pH 7.4, and in the remaining flasks 1, 2, 4 ml, and so on, of medium were substituted by the same amount of Kochsaft containing the same concentrations of substrate and acetate or of substrate alone. The tissue used in all the flasks was 2 g of cuttings of female rat livers, cut as homogeneous as possible. The flasks were gassed with oxygen and incubated at +37°C for 4 hours.

The results of the experiments are shown in Fig. 26, in which the amount of acetylated amine formed in 4 hours, expressed in μmoles on the ordinate, is plotted against units of CoA on the abscissa. Curve I is for the experiments made with 2 μmoles of

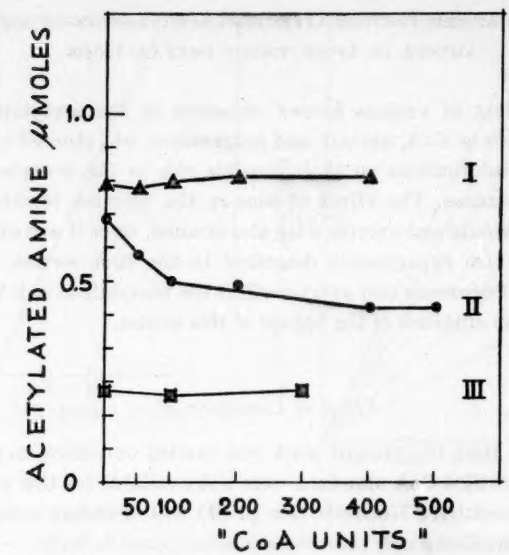


Fig. 26. — Effect of added CoA. Ordinate: amount of amines acetylated in 4 hours. Abscissa: CoA units of added liver Kochsaft. 2 g of liver tissue cuttings in 10 ml of standard medium, pH 7.4. Curve I: Experimental series with 2 μmoles of SAA and 200 μmoles of acetate. Curve II: Series with 2 μmoles of SAA but no acetate. Curve III: Series with 0.5 μmole of PAB and 200 μmoles of acetate. Gas phase: oxygen.

SAA and 200 μmoles of acetate, curve II shows the series containing 2 μmoles of SAA but no acetate, and curve III the series with 0.5 μmole of PAB and 200 μmoles of acetate.

It is seen from the graph that the addition of liver Kochsaft had no effect in the experimental series I and III. In series II, which contained no acetate, the added liver Kochsaft caused at its maximum inhibitory effect a decrease to 0.42 μmole in the amount of acetylated SAA, from 0.60 μmole in the control flasks.

Effect of Acetate and Cysteine

The results of the experiments described in Fig. 16, 21 and 26 already showed the effect of added acetate. In Fig. 16 the rate of reaction increased when the amount of acetate used was 100 times

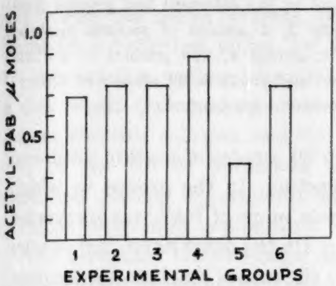


Fig. 27.

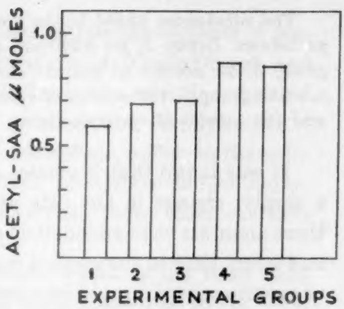


Fig. 28.

Fig. 27. — Effect of acetate and cysteine. Ordinate: amount of PAB acetylated in 4 hours. Substrate: 2 μmoles of PAB. 2 g of liver tissue cuttings in 10 ml of standard medium. pH 7.4. Incubation at 37°C. Columns 1—6: results of different experimental groups. Group 1: controls without acetate. Group 2: 2 μmoles of acetate. Group 3: 20 μmoles of acetate. Group 4: 200 μmoles of acetate. Group 5: 120 μmoles of cysteine. Group 6: 20 μmoles of acetate and 120 μmoles of cysteine.

Fig. 28. — Effect of ions. Ordinate: amount of SAA acetylated in 4 hours. 2 g of liver tissue cuttings per 2 μmoles of SAA in 10 ml of medium, pH 7.4. Columns 1—5: results of different experimental groups. Group 1: Krebs-Ringer solution without Mg · · used as medium. Group 2: physiological sodium chloride solution and 0.1 M potassium sodium phosphate buffer, 1:1. Group 3: 0.1 M potassium sodium phosphate buffer. Group 4: same medium as in group 1, with 230 μmoles of Mg · ·. Group 5: same medium as in group 3, with 230 μmoles of Mg · ·. Gas phase: oxygen.

as large as the amount of substrate. In Fig. 21 the addition of 100 μmoles of acetate per gramme of liver slightly increased the rate of reaction with substrate amounts lower than the saturation point, but the maximum rate of reaction was unchanged. In Fig. 26, acetate counteracted the inhibitory action of added liver Kochsaft.

The effect of acetate addition on the reaction was studied in numerous experimental series. Typical results of such series are shown in Fig. 27, in which the amount of PAB acetylated in 4 hours is given in μmoles on the ordinate. The series was divided into six groups, each group being represented by a numbered column in the graph.

All the series were made with 2 g of homogeneous rat liver cuttings, 2 μmoles of PAB as substrate, and standard medium, pH 7.4. Oxygen was used as the gas phase and all the groups were incubated at the same time in the same water bath at +37°C for 4 hours.

The substances added to the medium in the different test groups were as follows: Group 1, no addition; group 2, 2 μmoles of sodium acetate; group 3, 20 μmoles of sodium acetate; group 4, 200 μmoles of sodium acetate; group 5, 120 μmoles of cysteine; and group 6, 20 μmoles of acetate and 120 μmoles of cysteine. Group 1 served as a control.

It was found that 2 μmoles and 20 μmoles of acetate produced a similar change in the rate of reaction. In the groups to which these amounts were added, 0.05 μmole more of PAB was acetylated in 4 hours than in the control group. On the other hand, 200 μmoles of acetate caused a rapid increase in the rate of reaction, the amount of PAB acetylated in group 4 being nearly 0.20 μmole greater than that in group 1. The addition of 120 μmoles of cysteine produced a marked decrease in the rate, but 20 μmoles of acetate used with this amount of cysteine increased the rate of reaction to the same level as the addition of 20 μmoles of acetate alone.

In preliminary experiments cysteine was added to the reaction mixture for the protection of sulphydryl groups of coenzyme A, as recommended by Kaplan and Lipmann (1948). With a reaction mixture of pH 7.4 it was to be expected that no non-enzymic transfer of acetyl groups from acetyl CoA to cysteine would take place, as occurs at pH 8.0 (Tabor, Mehler and Stadtman 1953).

Effect of Ions in the Reaction Mixture

The effect of ions in the reaction mixture was studied by changing the proportions of stock solutions used in the Krebs-Ringer solution. The only finding concerning the rate of reaction in this experiment was the observation that it was accelerated by increasing amounts of phosphate buffer up to one-third of the volume of the solution. This favourable effect probably was ascribable merely to the fact that the buffering capacity of the mixture increased. Evidence of this was obtained when 0.05 M tris buffer, pH 7.4, was used instead of Krebs-Ringer solution and was substituted by increasing amounts of phosphate buffer, pH 7.4. The phosphate was found not to accelerate the reaction.

However, these experiments revealed that the buffering capacity of the original Krebs-Ringer solution was too low for use in experi-

ments with the tissue cuttings. In most of the experiments it was therefore substituted either by a mixture of Krebs-Ringer and 0.1 M potassium sodium phosphate buffer in the ratio 1: 1, termed in this report the *standard medium*, by a mixture of physiological sodium chloride solution and 0.1 M phosphate buffer in the ratio 1: 1, or by 0.1 M potassium sodium phosphate buffer alone. Numerous control experiments showed that these three reaction mixtures were fully comparable.

The »original» Krebs-Ringer solution, which is the term used in this report, contains 13 μmoles of magnesium per 10 ml. This amount of magnesium was found to have no effect on the rate of reaction. Only in very high — nearly 20-fold — concentrations was a definite effect seen.

The results of a series of these experiments are shown in Fig. 28, in which the ordinate gives the amount in μmoles of sulphanilamide acetylated in 4 hours. The figures in the abscissa refer to the experimental groups. The solutions tested in the different groups were as follows: Group 1, Krebs-Ringer solution, pH 7.4, without magnesium; group 2, physiological sodium chloride solution and 0.1 M potassium sodium phosphate buffer, 1: 1; group 3, 0.1 M potassium sodium phosphate buffer; group 4, same medium as in group 1, with 230 μmoles of magnesium added; group 5, same medium as in group 3, with 230 μmoles of magnesium added.

In these experiments, also 2 g of rat liver cuttings were used per 2 μmoles of sulphanilamide in 10 ml of medium. Oxygen was used as the gas phase and the flasks were incubated at +37°C for 4 hours.

Examination of the results shows that, compared with group 1, 0.1 μmole more amine was acetylated in phosphate buffer in groups 2 and 3. The addition of 230 μmoles of magnesium produced an increase of about 40 per cent in the rate of reaction. This was not potentiated further by phosphate.

Effect of Benzoate and Avertin

In the *in vivo* experiments in the first part of this report (Fig. 11 and 12, pp. 36 and 38) it was observed that benzoate and avertin (tribromoethanol) increased the amount of acetylated conjugates of PAB excreted in the urine. Their effect in the organism, however, could not be directly compared with the data obtained in urine analyses, for the rate of excretion of PAB was found to

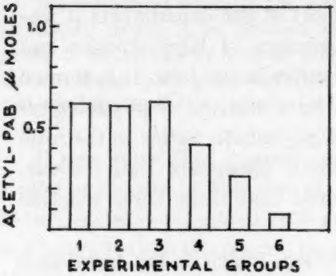


Fig. 29.

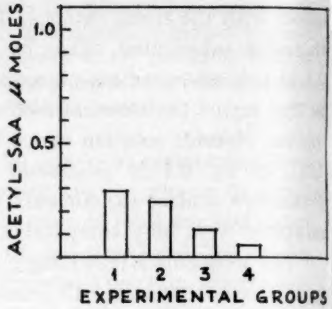


Fig. 30.

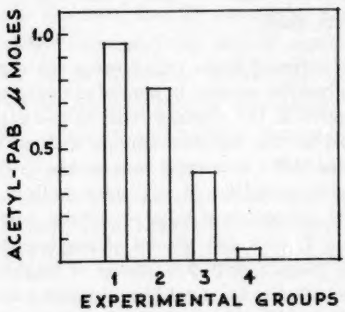


Fig. 31.

Fig. 29. — Effect of benzoate. Ordinate: amount of PAB acetylated in 4 hours. 2 g of liver tissue cuttings per 2 µmoles of PAB in 10 ml of standard medium containing 200 µmoles of acetate, pH 7.4. Columns 1—6: experimental groups. Group 1: controls, no benzoate added. Group 2: 1 µmole of benzoate added. Group 3: 2 µmoles of benzoate. Group 4: 10 µmoles of benzoate. Group 5 : 20 µmoles of benzoate. Group 6: 200 µmoles of benzoate. Incubation temperature 37°C. Gas phase: oxygen.

Fig. 30. — Effect of benzoate. Ordinate: amount of SAA acetylated in 4 hours. 2 g of liver tissue cuttings in standard medium, pH 7.4, containing 200 µmoles of acetate and 0.5 µmole of SAA per 10 ml of medium. Columns 1—4: experimental groups. Group 1: controls, no benzoate. Group 2: 2 µmoles of benzoate. Group 3: 20 µmoles of benzoate. Group 4: 200 µmoles of benzoate. Gas phase: oxygen.

Fig. 31. — Effect of benzoate and avertin. Ordinate: amount of PAB acetylated in 4 hours. 3 g of liver tissue cuttings in standard medium, pH 7.4, containing 300 µmoles of acetate and 3 µmoles of PAB per 15 ml of medium. Columns 1—4: experimental groups. Group 1: controls, no additions. Group 2: 30 µmoles of avertin. Group 3: 30 µmoles of benzoate. Group 4: 30 µmoles of avertin and 30 µmoles of benzoate. Incubation temperature: 37°C. Gas phase: oxygen.

undergo a change at the same time. With a change in the rate, the extent of PAB acetylation naturally also changed, the acetylation increasing as the excretion decreased, and decreasing as the excretion increased.

Experiments *in vitro* do not contain this considerable source of error of the *in vivo* experiments. It was therefore considered to be of interest to study *in vitro* the effect of benzoate on the acetylation of PAB and to compare it with the effect on the acetylation of SAA.

In the *in vitro* experiments the addition of benzoate to rat liver cuttings which then were incubated in an oxygen atmosphere with either PAB or SAA clearly decreased the acetylation of both amines if benzoate was used in more than 4-fold amounts compared to the amount of substrate. This effect increased with increasing amounts of benzoate, until 100-fold amounts of benzoate, compared to the amount of substrate, caused a nearly 80 per cent inhibition of acetylation, and larger doses inhibited it almost completely.

Fig. 29, 30 and 31 show the results of experiments performed to study the inhibitory action of benzoate on the acetylation of PAB and SAA. The amount of acetylated amine formed in 4 hours is shown on the ordinate.

In Fig. 29, no additions were made in test group 1. One μmole of benzoate was added in group 2 but did not appear to cause any change. No change was seen in group 3, either, to which 2 μmoles of benzoate were added. In group 4, 10 μmoles of benzoate produced definite inhibition of the reaction. In group 5, 20 μmoles of benzoate were used, causing further increase of the effect, until in group 6, containing 200 μmoles of benzoate, nearly 80 per cent of the acetylation was inhibited.

The experiments shown in Fig. 30 were also made with 2 g of rat liver cuttings, using the same medium as in the preceding experiment but only 0.5 μmole of sulphanilamide. No addition was made in group 1. The addition of as little as 2 μmoles of sodium benzoate (group 2) clearly depressed the rate of reaction. In group 3 the addition of 20 μmoles of benzoate enhanced further the inhibitory action, and in group 4, in which 200 μmoles of sodium benzoate were used, only 0.05 μmole of sulphanilamide was acetylated. We observe from this that when decreasing amounts of

substrate but a constant amount of liver tissue were used, already small amounts of benzoate caused inhibition, whereas the percentage of inhibition by large amounts of benzoate decreased.

Together with the effect of benzoate on the acetylation of PAB, the effect of avertin was also studied. The columns in Fig. 31 show the results of a series of experiments made with these additions. The ordinate is the amount in μmoles of PAB acetylated in 4 hours. In group 1, represented by the first column, no additions were made, 30 μmoles of avertin were used in group 2, 30 μmoles of benzoate in group 3, and 30 μmoles of avertin and 30 μmoles of benzoate in group 4.

The amount of avertin used in group 2 inhibited the acetylation by 21 per cent and that of benzoate in group 3 by 49 per cent, while avertin and benzoate used together in group 4 inhibited 94 per cent of the acetylation. The additive effect of the two substances was therefore greater than the sum of their separate effects.

DISCUSSION OF THE *IN VITRO* EXPERIMENTS

The results of the *in vitro* experiments described above clearly indicated that conjugation in rat liver and kidney cuttings occurs at the amino group of *p*-aminobenzoic acid and sulphanilamide. The conjugation is clearly an enzymic one and under the test conditions used it is bound to the intact cell, for only low or no activity was demonstrable in homogenates made from the same quantity of tissue. Furthermore the phenomenon had also other features characteristic of enzymic reactions.

The disappearance of activity observed after homogenization of the tissue may be due to a number of causes. Under the present experimental conditions the acetyl coenzyme A may be consumed by other processes that utilize it, or it may disappear either by deacetylation (Gergely, Hele and Ramakrishnan 1952) or by splitting of the coenzyme A itself. As is well known, the CoA molecule can be destroyed by tissue autolysis (Lipmann 1945). It has also been possible to cause degradation of CoA by several enzymes (*e.g.*, Lipmann, Kaplan, Novelli, Tuttle and Guirard 1947, Novelli, Kaplan and Lipmann 1949, 1950).

It also may be regarded as possible that with the breaking of the cells the deacetylated enzymes are activated and the formed acetylated aromatic amines are deacetylated (KOHL and FLYNN 1940, KREBS *et al.* 1947).

There may be a possibility, however, that the amount of acetyl CoA necessary for the acetylation of aromatic amines is not formed in homogenates, or that the acetylating enzymes are more readily inactivated during incubation in the broken than in the intact cell.

On the basis of earlier experimental results, the liver is considered to be the principal site of acetylation of aromatic amines in the organism (HARRIS and KLEIN 1938, KLEIN and HARRIS 1938, STEWART *et al.* 1939, Krebs *et al.* 1947, ANKER 1950, PHILLIPS and ANKER 1957, and others; cf. also the section on this subject in the review of the literature, pp. 13—14).

In the present experiments, however, the most marked acetylating activity per gramme of tissue was regularly observed in the kidney of the male rat when *p*-aminobenzoic acid was used as substrate. This divergence of the results from those obtained in many of the earlier investigations may be caused by the greater thermolability of the activity in the kidney, or possibility by a large excess of acetate in the reaction medium used. The observation has been made that under aerobic conditions acetate rapidly disappears from the renal cortex (ELLIOT and SCHROEDER 1934, ELLIOT, BENOY and BAKER 1935).

Earlier investigations have already shown that acetate is the most potent acetyl donor in the acetylation of aromatic amines (KLEIN and HARRIS 1938, LIPMANN 1945, KINNUNEN 1946). In the present study, equal and 10-fold amounts of acetate as compared to the amount of substrate clearly produced the same, though small, increase in the rate of reaction, whereas 100-fold amounts increased the rate considerably. It does not seem possible that the need of acetate would be so great, for the additional amount of aromatic amine acetylated under these conditions utilized only about 1/1000 of the acetate offered to it. As is well known, acetate thiokinase (aceto-CoA-kinase) has the greatest affinity for acetate (HELE 1954). It may, therefore, be considered possible that the large amounts of acetate used in the present experiments may have,

in addition to activating acetate thiokinase (aceto-CoA-kinase), replaced other acyl groups in the acyl CoA. Besides acetyl CoA, also other acyl coenzymes A participate in the aryl amine acetylation, since the enzyme which acetylates amines is not absolutely specific for the acetyl group but may also transfer other acyl groups, for example succinyl (SANADI and LITTLEFIELD 1951), from CoA to the aromatic amines. The rate of reaction, however, decreases with increasing carbon atom numbers of the acyl group, and palmityl CoA has an inhibitory action on the enzyme (TABOR *et al.* 1953). In consideration of these facts, it would appear probable that the increase in the rate of reaction produced by large amounts of acetate is in part, at least, a result of the disappearance of reaction-inhibiting factors.

When about 2 μmoles of CoA were added in the form of liver Kochsaft, the acetylation of *p*-aminobenzoic acid in liver cuttings was inhibited about 40 per cent. The inhibitory action of CoA has been shown also earlier, when it was observed that a 2-fold amount of CoA as compared to the amount of acetyl CoA produced 50 per cent inhibition (TABOR *et al.* 1953, TABOR 1955). The addition of an equal amount of CoA together with the acetate had neither an inhibitory nor an activating effect on the reaction. The absence of inhibition is probably due to the conversion, in the presence of a 100-fold amount of acetate, of all the CoA in the rat liver cuttings to acetyl CoA, of which there would thus be about 2 μmoles. This amount of acetyl CoA is a large one when we consider that 2 g of rat liver contain only about 1 μmole of CoA (LIPMANN 1950). Assuming that this process actually took place, and taking into consideration that the amount of acetylated aromatic amine formed did not increase, it would seem possible that there was available in the liver cuttings during 4 hours' incubation an amount of acetyl CoA sufficient for the acetylation of the aromatic amine offered to it in these experiments.

The differences in the activity of liver and kidney, also, were probably not due to a difference in the CoA content, since the kidney was able to acetylate nearly two times as much amine per gramme of tissue as the liver. Nevertheless the CoA contents of these two tissues showed an inverse ratio, for while the liver con-

tains about 0.5 μmole of CoA per gramme, the kidney contains only 0.3 μmole per gramme (LIPMANN 1950).

In the same manner as the CoA, cysteine also caused a decrease in the amount of aromatic amine acetylated if acetate was not added with the cysteine; when cysteine and acetate were given at the same time this inhibition was not seen. Even though the pH of the reaction mixture was only 7.4, one of the reasons for the inhibitory action may be a non-enzymic reaction in which the acetyl group is transferred from acetyl CoA to the -SH group of cysteine. STADTMAN (1952) has demonstrated the non-enzymic transfer of the acetyl group from acetyl CoA to cysteine, thioglycolate and glutathione. TABOR *et al.* (1953) observed that this non-enzymic reaction interferes with the acetylation of aromatic amines if acetyl CoA is used instead of CoA. By the same mechanism cysteine affects the synthesis of hippurate when benzoyl CoA and glycine are used as substrates, in which case there occurs a transfer of the benzoyl group to cysteine (SCHACHTER and TAGGART 1953). When acetate was added with the cysteine in the present experiments, the loss of acetyl CoA was probably compensated by a new synthesis of acetyl CoA.

In this work benzoate was found to potently inhibit the acetylation of PAB and SAA by liver tissue cuttings. This finding is in agreement with reports in the literature, for as early as in 1935 JOWETT and QUASTEL showed that benzoate inhibits the oxidation of butyric acid and crotonic acid by liver slices (1935 a, b). Inhibition of the oxidation of acetoacetic acid was also observed by the same group of workers (QUASTEL and WHEATLEY 1935). It has been demonstrated that the activation of acetate (JONES *et al.* 1953), fatty acids (KORNBERG and PRICER 1953, MAHLER, WAKIL and BOCK 1953) and acetoacetic acid (STERN, COON and DEL CAMPILLO 1953) requires adenosine triphosphate and CoA. Benzoate, also, needs adenosine triphosphate and CoA for its activation (CHANTRENNE 1951). Thus benzoate appears to compete with acetate for both CoA and adenosine triphosphate, which readily explains the inhibition which it produces. The apparent competitive inhibition when small amounts of aromatic amine are used as substrate is probably not a sign that benzoate inhibits also the arylamine

acetylase (aceto-arylamine-kinase) reaction. The reason would rather seem to be that, with decreasing amounts of the aromatic amine, there is competition for acetyl CoA, the availability of which is limited in the presence of benzoate by some other substrate which utilizes it, for example oxaloacetate. Large amounts of aromatic amine, again, probably have an inhibitory action on reactions of other substrates. KINNUNEN (1946) demonstrated in this laboratory that sulphapyridine has an inhibitory action on the formation of citric acid *in vitro* in rabbit liver pulp, and that malate inhibits the acetylation of sulphapyridine under the same conditions.

Avertin, as is well known, is a narcotic. Its effect on acetylation is probably based on this fact, for narcotics cause inhibition as well of the respiration of tissue as of arylamine acetylation in tissue homogenates. If adenosine triphosphate is added to the latter the acetylation-inhibiting action of the narcotic is eliminated. It seems, as had been suggested (JOHNSON and QUASTEL 1953 a, b), that narcotics possibly inhibit also the synthesis of adenosine triphosphate. The inhibition of adenosine triphosphate synthesis would serve to explain the present finding that avertin and benzoate when used together caused more marked inhibition than the sum of the separate inhibitions of the two substances.

The lack of activating power of magnesium on acetylation observed in the present experiments is probably due to its specific effect on the acetate thiokinase (aceto-CoA-kinase) reaction only (CHOU and LIPMANN 1952). The effect of magnesium on acetylation may therefore be overshadowed, since the amount of acetyl CoA formed in acetate activation is only a part of the total formation of acetyl CoA in the liver.

EEFECT OF THE SEX GLANDS ON THE AROMATIC AMINE ACETYLATING ACTIVITY *IN VITRO*

It was observed in the preceding section of this study that in using homogeneous tissue the conjugation at the amino group of PAB was directly proportionate to the amount of tissue in the reaction mixture. This made it possible to compare the acetylating activity of similar tissues of different animals of the same species

by using equal amounts (by weight) of the tissues to be compared, and by determining the acetylated *p*-aminobenzoic acid formed under identical experimental conditions.

In this section of the investigation a comparison was made of the acetylating activity *in vitro* of the tissues of male, castrated male, female and castrated female rats of different age groups by determining the amount of acetyl-PAB formed within a given time by the same amounts of tissue. The tissues tested were liver and kidney, since conjugation to the amino group of PAB had been regularly observed in both tissues *in vitro*.

The standard experimental conditions used were based on the experience gained in the preceding section of this work. Because of its special nature the experimental technique is described below.

SPECIAL EXPERIMENTAL TECHNIQUE

The same reaction medium was used in all the experiments in this section. This medium, as well as the buffer used in it, was prepared immediately before each experiment from ice-cold stock solutions as follows:

Physiological sodium chloride solution	200	ml
0.154 M potassium chloride solution	8	»
1.0 M sodium acetate solution	8	»
0.02 M PAB solution	4	»
0.1 M potassium sodium phosphate buffer, pH 7.4, to make	400	ml

Of this medium was used 10 ml per 2 g of liver or per kidney, giving 2 μmoles of substrate and 200 μmoles of acetate in both cases. Each experimental series comprised four rats, *i.e.*, one male, one castrated male, one female and one castrated female rat. From each rat samples were taken into four incubation flasks, in two of which there were 2 g of liver and in the remaining two flasks one kidney in each. Cuttings were prepared of these tissues in the flasks as previously described. One series of experiments was carried out at a time, the entire series being prepared simultaneously. All the flasks in a series were gassed with oxygen, placed in the water bath at the same time, and incubated at +37°C for 4 hours. Care was taken to keep the speed of shaking similar in all the series. After incubation, two samples of 1 ml were drawn from each flask into a centrifuge tube containing 4 ml of 5 per cent trichloroacetic acid. The samples were immediately placed in the refrigerator, where they were kept until analysed.

TABLE 2

AMOUNTS OF ACETYL-PAB IN MICROMOLES FORMED IN LIVER AND KIDNEY TISSUE CUTTINGS FROM DIFFERENT EXPERIMENTAL ANIMAL SERIES, SHOWING EFFECT OF SEX AND CASTRATION ON THE ACETYLATING ACTIVITY

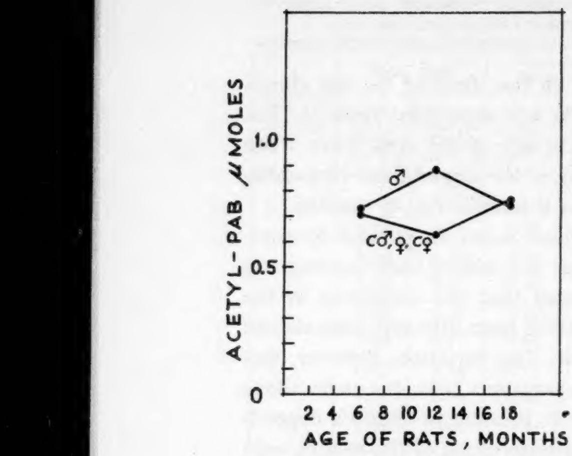
Mean Age of Rats, Months	Male Rats		Castrated Male Rats		Female Rats		Castrated Female Rats	
	Liver Acetyl-PAB per 2 g	Kidney Acetyl-PAB per 1 g	Liver Acetyl-PAB per 2 g	Kidney Acetyl-PAB per 1 g	Liver Acetyl-PAB per 2 g	Kidney Acetyl-PAB per 1 g	Liver Acetyl-PAB per 2 g	Kidney Acetyl-PAB per 1 g
	0.62	0.67	0.74	0.54	0.76	0.37	1.06	0.55
	0.54	0.67	0.78	0.54	0.98	0.35	1.06	0.46
	0.56	0.58	0.68	0.67	0.67	0.40	0.61	0.40
	0.56	0.57	0.68	0.75	0.66	0.41	0.64	0.31
	0.66	0.38	0.72	0.51	0.72	0.20	0.48	0.43
18	0.85	0.37	0.81	—	0.64	0.11	0.60	0.30
	0.83	0.54	0.75	0.65	0.93	0.23	1.12	0.52
	0.90	0.64	0.73	0.58	0.82	0.26	1.04	0.37
	1.00	0.69	0.60	0.47	0.72	0.32	0.78	0.36
	0.88	0.55	0.49	0.52	0.76	0.38	0.68	0.50
	1.06	0.84	0.50	0.67	0.77	0.37	0.72	0.46
	1.06	0.76	0.61	0.68	0.79	0.28	0.58	0.51
	0.90	0.81	0.50	0.51	0.60	0.23	0.90	0.47
	0.86	0.95	0.56	0.59	0.67	0.39	0.91	0.32
	1.15	0.92	0.75	0.55	0.61	0.38	0.86	0.38
	1.09	0.80	0.64	0.36	0.99	0.31	0.86	0.40
	0.75	0.76	0.66	0.59	0.61	0.33	0.66	0.36
	0.83	0.83	0.64	0.54	0.63	0.34	0.60	0.43
	0.71	0.58	0.61	0.48	0.45	0.32	0.46	0.54
12	0.71	0.60	0.47	0.54	0.50	0.27	0.47	0.58
	0.68	0.56	0.24	0.58	0.42	0.55	0.52	0.68
	0.70	0.79	0.48	0.63	0.42	0.45	0.40	0.61
	0.84	0.93	0.52	0.44	0.88	0.37	0.42	0.34
	0.86	0.76	0.56	0.48	0.70	0.43	0.50	0.42
	0.93	0.82	0.59	0.65	0.68	0.49	1.01	0.72
	0.92	0.87	0.75	0.59	0.76	0.37	0.81	0.66
	0.77	0.90	0.65	1.00	0.62	0.33	0.44	0.59
	0.96	0.78	0.85	0.72	0.48	0.20	0.37	0.43
	0.75	0.93	0.77	0.84	0.74	0.58	0.54	0.37
	0.85	1.01	0.76	0.88	0.59	0.64	0.48	0.39
	1.04	0.71	0.57	0.67	0.86	0.43	0.73	0.63
	0.96	0.83	0.63	0.69	1.14	0.27	1.01	0.72
	0.53	0.87	0.82	0.97	0.50	0.47	0.60	0.37
6	0.54	0.77	0.81	0.79	0.48	0.37	0.65	0.46
	0.58	0.89	0.62	0.91	0.90	0.27	0.59	0.48
	0.54	0.96	0.61	0.72	0.92	0.53	0.64	0.70
	0.77	1.05	—	—	—	—	0.73	0.62
	0.71	1.02	—	—	—	—	0.86	0.73

RESULTS

The results of the experiments on the effect of the sex glands on the acetylating activity of rats are shown in Table 2. The animals were from three litters, the age of the first litter when killed being 18 months (± 1 month), of the second litter 12 months (± 1 month) and of the third litter 6 months (± ½ month).

The mean values for each animal based on parallel determinations of duplicate samples of liver and kidney tissue cuttings are stated in the table. It was observed that the variations in the activity of tissue preparations obtained from different animals and at different times were considerable. The variation, however, was very small between two tissue preparations from the same tissue and from the same animal. Therefore, in order to obtain a dependable quantitative comparison for interpretation of the results, only the experiments in the same series may be directly compared with each other. One experimental series, however, does not always give a reliable picture of differences in the acetylating activity of non-castrated and castrated rats of the two sexes, since only one rat of each kind was included in every series. Biological variations between the individual animals in a group produce differences between the various series. Variations in the external experimental conditions, however, were similar in all the groups. Since the effect of changes in the external experimental conditions is therefore equalized within each group, the results of the different experimental series may be combined for the purpose of comparison of the results of the different kinds of animals, *i.e.*, non-castrated and castrated males and females.

On examination of the mean values of acetyl-PAB formed in the different groups of rats it is observed that the mean value of the experiments made with the livers of non-castrated male rats in all the experimental series was 0.80 μmole of acetylated PAB per 2 g of liver, or markedly higher than that in the other groups of animals. The results for the other groups differed but little from each other. The mean for castrated male rats was 0.65 μmole, for non-castrated females 0.70 μmole, and for castrated females 0.69 μmole of acetyl PAB per 2 g of liver tissue.



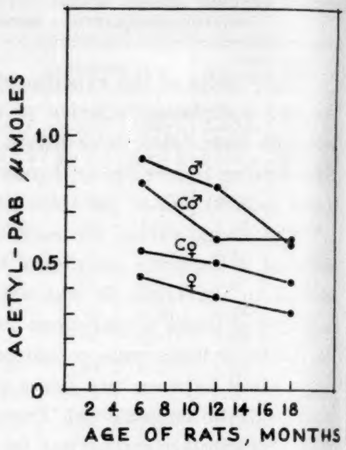


Fig. 32. Fig. 33.

Fig. 32. — Mean results of experiments with liver tissue in different age groups from Table 2. Ordinate: amount of PAS acetylated in 2 g of liver tissue cuttings in 4 hours. Abscissa: age of rats in months. Upper line: male rats. Lower line: castrated male, non-castrated female and castrated female rats.

Fig. 33. — Mean results of experiments with kidney tissue in different age groups from Table 2. Ordinate: amount of PAB acetylated in 1 g of kidney tissue cuttings in 4 hours. Abscissa: age of rats in months. Lines from the top downwards: Non-castrated male, castrated male, castrated female, and non-castrated female rats.

In reviewing the amounts of acetyl PAB formed in the same length of time in kidney tissue, attention is drawn to the finding that in the non-castrated and castrated male groups the amount was twice as large as that formed in the same amount of liver. Even in the less active non-castrated and castrated females the kidney tissue appeared to be more active than the liver tissue.

The effect of the sex glands and their removal on the acetylation comes out clearly when we compare the mean values for acetylation in the kidney tissue of the different kinds of animals. Using the same experimental time, one gramme of kidney of non-castrated male rats formed 0.76 μmole of acetylated conjugates of *p*-aminobenzoic acid, that of castrated male rats 0.64 μmole, of non-castrated females 0.36 μmole, and of castrated females 0.49 μmole.

Since differences were seen even within the groups in the mean values for rats of different age, both in the experiments with liver tissue and in those with kidney tissue, these mean values for acetyl PAB formed in the different age groups are shown in Fig. 32 and 33.

From Fig. 32, which gives the results of the experiment swith liver tissue, it is observed that when the three kinds of animals in whose acetylating capacity in the liver no statistical difference was seen are combined and compared with the male rats, no difference in the acetylating power is observed between the young rat groups, between the old rats groups, or between these two age groups. In the intermediate age group, on the other hand, a definite difference is seen. At this time, when the activity of the sex glands also is highest, the acetylating power of the non-castrated males increased. The decrease in the acetylating capacity of the other animals of the same age group increased the difference between the two sets of results.

The correlation between age and the acetylating activity of the kidneys differs from the above, as is seen in Fig. 33. With increasing age the activity decreased in all the age groups. The decrease was greatest in non-castrated males, and in the oldest age group there was no difference in acetylation between the non-castrated and castrated male rats. With castrated rats the kidney activity curve from the second to the third age group resembles the moderately oblique curves of the non-castrated and castrated female rats.

Table 3 shows the mean weights of body, liver and kidneys of the four kinds of animals whose tissues were tested, as well as the mean values obtained in the liver and kidney acetylation experiments *in vitro*, which already were listed in Table 2. On the last line is given the amount of acetyl-PAB formed in the liver and kidneys *in vitro* in 4 hours, expressed in μmoles per kilogram of body weight, after correction of the dosage to correspond to 100 μmoles per kilogramme of body weight in all the groups. Using the same manner of correction of the dosage, the amount of acetyl PAB calculated to have been formed *in vivo* in the different experimental groups in 4 hours were shown in Table 1 (p. 40).

The amount of PAB acetylated in μmoles per kilogramme of body weight was calculated as follows. It is assumed that the portion of liver not used in the experiments had the same activity as the portion used. The total acetyl PAB production of each liver was calculated on the basis of the weight of the liver and the amount of acetyl PAB formed by two 2 g samples of it. To the resulting amount in μmoles was added the acetyl PAB production of the two kidneys in μmoles. The total acetyl PAB amount was then calculated per kilogramme of body weight of the animal.

The dosage was calculated by multiplying the weight of the liver by 1 μmole (the dosage in the experiments with liver having been 2 μmoles of PAB per 2 g of liver tissue). The amount of PAB used for the two kidneys ($2 + 2 = 4$ μmoles) was added and the total dose was calculated per kilogramme of body weight and corrected for every animal to 100 μmoles per kg.

Using the correction coefficients obtained in calculating the dosage in 100 μmoles per kg, the total amount of acetyl PAB produced by each rat per kilogramme of body weight was converted to correspond to a dosage of 100 μmoles. The group means of these amounts are shown on the lowest line in Table 3.

TABLE 3

MEAN GROUP VALUES OF THE BODY AND ORGAN WEIGHTS, OF THE ACETYL-PAB FORMATION IN THE LIVER AND THE KIDNEY *in vitro*, AND OF THE CALCULATED TOTAL ACETYL-PAB FORMATION *in vitro*

	Male Rats	Castrated Male Rats	Female Rats	Castrated Female Rats
Weight of rats, g	288	266	182	233
Weight of liver, g	10.609	9.044	7.644	8.885
Weight of kidney, g	1.090	0.941	0.800	0.908
Acetyl-PAB formed per 2 g of liver, μmoles	0.80	0.64	0.70	0.69
Acetyl-PAB formed per g of kidney, μmoles	0.76	0.64	0.36	0.49
Acetyl-PAB formed *in vitro*, μmoles per kg of body weight, after correction of PAB dose to 100 μmoles per kg of body weight	41.5	30.5	28.1	31.3

Comparison of these *in vitro* values with those in Table 1 for the *in vivo* experiments reveals a good numerical correspondence between the results. The difference between the male and female

rats in acetylation was also of the same order of magnitude. The effect of castration of female rats was only slightly more marked, the increase in acetylating power being 9.1 per cent *in vivo* and 11.4 per cent *in vitro*. An unexpected observation was that, in the light of these results, the depressing effect of castration of male rats on acetylation increased three-fold from 7.4 per cent in the *in vivo* experiments to 26.5 per cent in the *in vitro* experiments.

STATISTICAL TREATMENT OF THE RESULTS

The statistical method used in most cases was the two-way variance analysis, in which the effect of two separate factors and of their interaction on the observed random variable was studied simultaneously.

Statistical analysis was first made of the differences between the experimental series. The two factors subjected to simultaneous study were the experimental series and sex. The interaction in the analysis of the difference between the series depicts the dispersion that would have occurred within the series if each series had comprised rats of one sex only.

The method was applied first to the liver acetylation experiments in the intermediate age group.

— It was found that the dispersion of the experimental series was highly significantly ($P < 0.001$) greater than the dispersion of parallel experiments, and significantly ($P < 0.01$) greater than the interaction. Similarly the interaction was significantly greater ($P < 0.01$) than the dispersion of the parallel experiments.

— The difference between the sexes in the acetylation in the liver was highly significant ($P < 0.001$) in the intermediate age group. It was later seen that this difference occurred between the male group and the other groups studied; between the latter three groups no differences were seen (cf. Fig. 32).

The differences between the experimental series in the kidney acetylation experiments were then analyzed statistically. The same method and factors of analysis were used as in the analysis of the liver acetylation experiments.

— The dispersion between these experimental series was highly significantly ($P < 0.001$) greater than the dispersion between the right and the left kidney of each rat, but it was of the same order as the interaction. The interaction thus is also highly significantly ($P < 0.001$) greater than the dispersion between the right and the left kidney. This may be interpreted to indicate that in rats of the same sex and age the acetylating power of the kidneys varies more greatly than that of the liver.

After statistical analysis of the differences between the experimental series, the liver and kidney acetylation experiments were studied by two-way variance analysis by using age and sex as the simultaneously effective factors. The marked interaction thus indicates that the change with age in the sex groups is different. In addition to carrying out the two-way variance analysis, the results obtained in the statistical analysis of the liver experiments were controlled by the *t*-test.

Analysis of the liver acetylation experiments by two-way variance analysis:

— No difference was observed between castrated male rats, female rats and castrated female rats.

— The change with age was significant at the level of $P = 0.04$.

Analysis of liver acetylation by the *t*-test:

— The acetylation in the liver of male rats of the intermediate age group was highly significantly ($P < 0.001$) greater than in the corresponding age group in the three other groups of animals.

— By the *t*-test were also compared the intermediate age group of male rats with the combined young and old age groups of males. The intermediate group acetylated more than the combined group, the difference being almost significant ($P < 0.05$).

The statistical results for the liver acetylation experiments may be interpreted to indicate that in male rats the change with age differs from that in the other test groups. Thus the acetylation of PAB in the liver of male rats was highest in the intermediate age group, while in castrated male, non-castrated female, and castrated female rats it was lowest in this age group. On the other hand, as was already seen from the mean values in Fig. 32, the difference in the acetylating power of the youngest and the oldest age groups of

male rats was not significant when these were compared with the other animals of the same age.

In studying the acetylation in the kidney by the two-way variance analysis, the comparison was made in three parts (cf. Fig. 33). The results of acetylation of PAB in the kidney were first compared in males and castrated males, then in castrated males and castrated females, and thirdly in castrated females and females. The factors thus were sex and age.

— PAB acetylation in males was highly significantly ($P < 0.001$) greater than in castrated males. Age also had a highly significant effect ($P < 0.001$). Since the interaction also was highly significant ($P < 0.001$), this is to be interpreted as indicating that the change with age was different in these two groups.

— In castrated males the PAB acetylation in the kidney was highly significantly ($P < 0.001$) greater than in castrated females. The effect of age was significant ($P < 0.01$) but the interaction was low. Accordingly, there was no statistical difference in the change with age in the two groups compared.

— Also castrated females had a highly significantly ($P < 0.001$) higher acetylating capacity for PAB in the kidney than females. Age, on the other hand, had an almost significant ($P < 0.05$) effect, and the interaction was small.

A statistical analysis was also made of the acetyl-PAB formation per kilogramme of body weight when using a dose corrected to 100 μmoles of PAB per kilogramme in the groups of males, castrated males, females and castrated females. On the basis of the mean values for the different groups (cf. Table 3) the analysis was made in two parts.

The first set of results examined were those of acetyl PAB formation by castrated male rats, females and castrated females, of which the mean values differed but slightly.

— In two-way variance analysis, using as factors sex and age, no significant differences were found between these groups in the acetylation of PAB. Age also had no significant effect (value obtained: $F = 2.47$, when $P = 0.05$ corresponded to $F = 3.20$).

Secondly the acetylating capacity of the male rats was analysed by comparing it with that of all castrated rats and of female

rats, and by comparing the different age groups of male rats by the t-test.

— In the intermediate age group of male rats the formation of PAB was highly significantly greater (obtained value: $t = 6.15$, when $P = 0.001$ corresponded to $t = 3.73$) than in a group formed by combining the intermediate age group of both castrated groups.

— In the intermediate age group of males the acetyl-PAB formation was also highly significantly greater (obtained value: $t = 6.89$, when $P = 0.001$, corresponded to $t = 3.73$) than in females of the same age group.

— The intermediate age group of male rats differed but slightly with regard to acetyl-PAB formation from the younger and older age groups of males, for in a comparison of the intermediate age group with the other groups the difference was significant only to the level of $P = 0.07$.

DISCUSSION OF THE EXPERIMENTS ON THE EFFECT OF THE SEX GLANDS

The reliability of the technique used in the experiments in this section may be evaluated by two criteria.

Statistical analysis of the dispersion of the results showed that the method used in the *in vitro* experiments gives satisfactory results. It may be regarded as an indication of the reliability of the results that the dispersion of the results of the duplicate samples in both the liver and the kidney experiments was smaller than the dispersion of the individual animals in a series if the series had consisted of rats of one sex only.

As a second criterion may be used a comparison of the quantities of acetyl-PAB conjugates formed *in vitro* and *in vivo*. Using as criterion the amount of acetyl-PAB formed in the rat per kilogramme of body weight we find that the average for all the experimental groups combined was only 7.1 per cent lower *in vitro* than *in vivo*. The uncorrected dosage of PAB per kilogramme of body weight in the *in vitro* experiments was, nevertheless, 23 per cent smaller than in the *in vivo* experiments. The similarity of the amount of PAB acetylated in the *in vivo* and the *in vitro* experiments is an indication that a considerable part of the acetylating

activity of the liver and the kidneys is retained in the *in vitro* experiments.

Differences in the acetylating activity of male and female rats were demonstrable also *in vitro*, for both the liver and the kidneys of male rats were capable of acetylating definitely larger amounts of PAB than the corresponding tissues of the females.

The results obtained indicate that the castration of male rats depresses the acetylating activity in the liver and the kidneys.

Non-castrated and castrated females showed a difference in the acetylating power of kidney tissue only, in which the castrated remales formed more acetyl-PAB than the non-castrated females. On the other hand, no difference was seen in the acetylating power of the liver tissue between these two groups of female rats, nor between either of these groups and castrated male rats.

On the basis of the results of the present experiments it appears probable that the male sex glands have a stimulating effect on the ability of liver tissue and kidney tissue to acetylate *p*-aminobenzoic acid. The female sex glands, on the other hand, seem to have an inhibitory action on the acetylating activity of the liver. This observation is supported by results obtained in experiments on the effect of testosterone, oestrogen and progesterone on the acetylation activity of liver and kidney tissues (LUUKKAINEN to be published).

Merely on basis of the *in vitro* experiments described above, conclusions cannot be drawn concerning the significance of the kidneys as a site of acetylation of PAB in the living organism. However, examination of the results of the *in vivo* experiments also shows differences in the PAB acetylating power *in vivo* of young and old male rats as compared to female rats of the corresponding ages (Fig. 3 and 7). The results of the *in vitro* experiments with liver reveal no differences in the acetylation activity of these age groups of male rats as compared to the female rats. Including the experiments with kidney tissue, the results of the *in vivo* and the *in vitro* experiments are in agreement. This may probably be regarded as evidence that also in the living organism the kidney has even an important rôle as a site of acetylation of *p*-aminobenzoic acid.

COENZYME A DETERMINATIONS

In planning the *in vitro* experiments, differences in the coenzyme A content were taken under consideration as one of the causes of possible differences between the sexes in the acetylation. To study this point, samples for CoA determinations were taken from the livers to be used in the *in vitro* experiments. It was not possible to obtain samples from kidneys for this purpose.

RESULTS OF THE CoA DETERMINATIONS

The results are shown in Table 3. In the first column are stated the numbers of the *in vitro* experiment groups from which these liver samples were taken. The average CoA contents per gramme of fresh liver are in the second column for male rats, in the third column for castrated male rats, in the fourth column for female rats, and in the fifth column for castrated female rats. Each result given is the mean of duplicate determinations of three dilutions. In the section describing the method of CoA determination it already was stated that the values are only relative owing to the technique and the own standard used. As is seen from the table,

TABLE 4

COENZYME A CONTENTS OF LIVERS, IN UNITS PER GRAMME OF LIVER TISSUE

Number of Experimental Series	Male CoA U./g	Castrated Male CoA U./g	Female CoA U./g	Castrated Female CoA U./g
1	157	179	168	165
2	99	97	100	128
3	122	127	135	133
4	142	119	146	142
5	130	138	122	139
7	145	143	120	119
10	109	137	145	124
12	154	171	174	152
13	110	121	137	132
Mean	130	137	139	137

the following mean CoA contents were obtained in the different groups: Male rats, 130 units; castrated male rats, 137 units; female rats, 139 units; and castrated female rats, 137 units, all per gramme of fresh liver.

DISCUSSION OF THE CoA DETERMINATIONS

On examination of the results, attention is drawn to the finding that the dispersion of the different groups within a series is smaller than the dispersion of series tested at different times. The large differences seen between the various series are probably not a result of differences in the CoA contents of the animals in a group, but rather of variations in the conditions during preparation of the specimens and of the Kochsaft. Since all the tested groups were represented in each experimental series every time, they were subject to the same external conditions. The mean values of the groups may therefore be considered to be comparable. It is seen from these results that no differences were demonstrable in the CoA contents in male, castrated male, female and castrated female rats. According to these results, therefore, differences in the acetylating activity cannot be explained on the base of differences in the coenzyme A content.

In different metabolic states in which there is a definite deficiency of CoA, the arylamine acetylation activity is, obviously, depressed. Animals kept on a pantothenic acid deficient diet and having a less than normal availability of CoA (Olson and Kaplan 1948, Klein and Lipmann 1953) acetylate less arylamines than normal animals (Riggs and Hegsted 1948, 1949, 1951, Shils *et al.* 1949, 1950; Riggs and Christensen 1951; Dumm and Ralli 1951).

A reduced capacity for the acetylation of aromatic amines similar to that in pantothenic acid deficiency is also seen in alloxan-diabetic rats (Charalampous and Hegsted 1949). This apparently is due to a non-enzymic inactivation by alloxan of a portion of CoA, similar to the inactivation of other SH compounds (Lazarow and Cooperstein 1953). The true CoA content, however, is increased in alloxan-diabetic rats (Wieland, Reinwein and Lynen 1956).

On the other hand, the aromatic amine acetylating power in

animals with an adequate supply of CoA no longer seems to be dependent on the CoA content. In hyperthyroidism the arylamine acetylation activity of rats is clearly decreased (Fraenkel-Conrat and Greenberg 1946), whereas thyroxine increases the CoA content (Tabachnick and Bonnycastle 1953, 1954; Ringler and Leonard 1954).

Similarly, deficiency of riboflavin also has resulted in some decrease in the extent of acetylation of aromatic amines (Riggs and Hegsted 1949). However, the CoA content of various tissues from riboflavin deficient animals is not different from that of non-deficient controls (Olson and Kaplan 1948).

Ringler and Leonard (1954) reported that the CoA content of the liver of hypophysectomised rats is lower than that of non-operated rats. Chernick and Moe (1956) were unable to confirm this observation, for in their investigation operated and non-operated rats of the same weight had the same CoA content, while control rats of the same age but of a heavier body weight had a lower CoA content per gramme of liver tissue than the operated rats.

Since hypophysectomy and, according to the results presented in this report, castration cause no change in the CoA content of the liver, the conclusion is probably justified that the difference observed between the sexes in the acetylating activity apparently is not due to a different CoA content. The slightly lower average content in male rats probably reflects merely the fact that the rats in this group have a higher body weight and therefore a larger liver.

GENERAL DISCUSSION

It is a well known fact that several species of animals are capable of conjugating the acetyl group to the aromatic amine that is administered to them. It is, however, still open to question why the animal organism acetylates these substances, foreign to it in amount or kind. The purpose may possibly be a more rapid elimination of amines rather than an actual detoxication process. There is, however, almost complete knowledge of the mechanism of this reaction as well as of some of its factors.

The elucidation of the mechanism of the reactions connected with the acetylation of aromatic amines is closely related to the study of acetic acid metabolism. In addition to the aromatic amine, there participates in the reaction the »active acetate», in the formation of which coenzyme A is needed as activator. From coenzyme A and acetyl precursors the organism forms in different ways active acetate, or acetyl coenzyme A, which has been shown to be the acetyl thio ester of coenzyme A. In the reaction catalysed by arylamine acetylase (aceto-arylamine-kinase), acetyl CoA then donates its acetyl group to the aromatic amine. The quantity of acetylation is therefore influenced by all the factors that enter into the formation and utilization of acetyl CoA, but it also is influenced by factors affecting the activity of arylamine acetylase.

When the central reactions that influence or are closely related to the formation of acetyl CoA are disturbed, as occurs in thiamine deficiency (MARTIN and RENNEBAUM 1943, RIGGS and HEGSTED 1949) and riboflavin deficiency (RIGGS and HEGSTED 1949), the ability of animals to acetylate aromatic amines is depressed from the normal.

As a product of its metabolism, a normal animal forms a large amount of acetic acid — about 1 per cent of its body weight in

24 hours (BLOCH and RITTENBERG 1945). Therefore, when an adequate dosage of arylamine is administered to the animal, simultaneous doses of acetyl precursors have been found not to affect the extent of acetylation (CHARALAMPOUS and HEGSTED 1949).

Accurate knowledge of the site of action of the hormones that influence aromatic amine acetylation is still lacking. It is known that in thyroid hyperfunction the excretion of acetyl conjugates is reduced (FRAENKEL-CONRAT and GREENBERG 1946, GERSHBERG *et al.* 1950, GERSHBERG and KUHL 1950, VAVREČKA and PETRÁŠEK 1955). In diabetes, data even on the direction of the process are contradictory. The existence of a difference in the acetylating power of male and female animals has been demonstrated by a number of investigators (KINNUNEN 1946, DUMM and RALLI 1951, FRANZ and LATA 1953, 1957). On the basis of the experiments carried out in the present investigation it appears probable that the sex glands of the male rat have the effect of increasing the acetylation activity both in liver tissue and in kidney tissue. The female sex glands of the rat, on the other hand, appeared to depress acetylation in kidney tissue only. The experiments described in this report, which seek to elucidate the course of the reaction, appear to suggest that the effect of the sex glands is concentrated directly on the activity of arylamine acetylase.

Investigation concerning factors influencing the acetylation of aromatic amines is inconvenienced by the difficulty of obtaining correct interpretations of the results of *in vivo* experiments because of the numerous sources of error associated with this experimental procedure. On examination of the experiments in this report it is observed that the administration of, for example, benzoate had a seemingly increasing effect on acetylation *in vivo*, whereas in the *in vitro* experiments it was a potent inhibitor.

Unless there is a definite deficiency of coenzyme A, determinations of this substance will not give a correct view of the arylamine acetylation activity. Furthermore it appears uncertain whether observation of the process of acetylation of aromatic amines will provide adequate information on the acetyl coenzyme A balance or on its formation and utilization.

SUMMARY

The object of the present investigation was to study the effect of the sex glands and their removal on the capacity of the rat to acetylate *p*-aminobenzoic acid. The investigation was carried out in three sections.

Experiments in vivo were made to study, in addition to the effect of sex and castration, also the effect of experimental conditions, fasting, avertin (tribromoethanol) and benzoate on the ability of rats to acetylate PAB.

Experiments in vitro, using oxygen as the gas phase in incubation at + 37°C, were made to study factors that may influence the acetylating activity for arylamines in the liver and the kidneys. When the optimum conditions for the acetylation of PAB had been found, the effect of sex and castration on this acetylation in rat liver and kidney tissue were studied.

Coenzyme A determinations were made by analysing *in vitro* the CoA contents of the livers of non-castrated and castrated male and female rats.

The results of the *in vivo experiments* showed that even among rats of the same weight the males excreted in the urine a larger amount of acetylated PAB conjugates than the females during experimental times of 4 hours and 24 hours and after doses ranging from 3 μmoles to 60 μmoles of PAB. Since the rate of excretion of PAB can be well followed during 4 hours, this period was selected for the subsequent experiments. With a dose of 15 μmoles of PAB, fasting for 72 hours was found to have no effect on the acetylating power of male or female rats as compared to control rats. When this dosage of PAB was combined with benzoate and/or avertin, the

percentage of acetylated PAB conjugates excreted in the urine was increased. A closer study of the effect of benzoate showed that it reduced the rate of excretion of PAB. This probably provides the explanation for the effect of benzoate *in vivo*. The castration of male rats slightly reduced their acetylating power as compared to non-castrated males. The excretion of actylated PAB conjugates by castrated female rats was higher than that by non-castrated female rats. The results of these experiments are discussed, attention being paid to the possible sources of error in experiments *in vivo*.

In the *in vitro experiments* it was found that minute tissue pieces, cuttings, were capable of converting more PAB to acetylated forms than tissue slices and homogenates. More acetyl PAB was formed also when oxygen instead of air was used as the gas phase. PAB and SAA were similarly acetylated when used in the same molar concentration in the optimum range of pH 7.0—7.8. In an acetate-containing reaction solution, twice as much acetyl PAB was formed in kidney tissue cuttings of male rats as in the same amount of liver tissue cuttings under the same conditions. Activation of a deacetylating enzyme was not observed in either of these tissues. The addition of acetate to the reaction mixture produced a definite but, in relation to the addition, small molar increase in the formation of acetyl PAB. Coenzyme A added in the form of Kochsaft and added cysteine caused inhibition of the acetylation, but if acetate was added at the same time there was no inhibition. Benzoate and avertin had an inhibitory action on the acetylation of PAB and SAA even in the presence of acetate. The discussion in the first section of the *in vitro* experiments deals with these observations and with reactions connected with the acetylation of aromatic amines.

The results obtained in the second section of the *in vitro experiments* showed that a larger amount of PAB was acetylated in the liver and kidney tissues of male rats than of female rats. Castration of male rats reduced the acetylating activity in both the liver and the kidneys. Castration of female rats increased the acetylation in kidney tissue. On the other hand, no difference was observed in the acetylating activity in the liver tissue of castrated male rats and non-castrated and castrated female rats. The results in this

section were analysed statistically, and the technique used and the significance of the kidneys as a site of acetylation of PAB also *in vivo* are discussed.

In *coenzyme A determinations* no difference was found in the CoA contents of the livers of male, castrated male, female and castrated female rats. The results are discussed, commenting on the CoA content and the aromatic amine acetylation in different metabolic states.

Factors affecting the acetylation of aromatic amines, the results obtained in the present investigation, and the interpretation of results of studies in the acetylation of aromatic amines in general are discussed.

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